(54) Title: GOLD-BINDING PROTEIN AND USE THEREOF
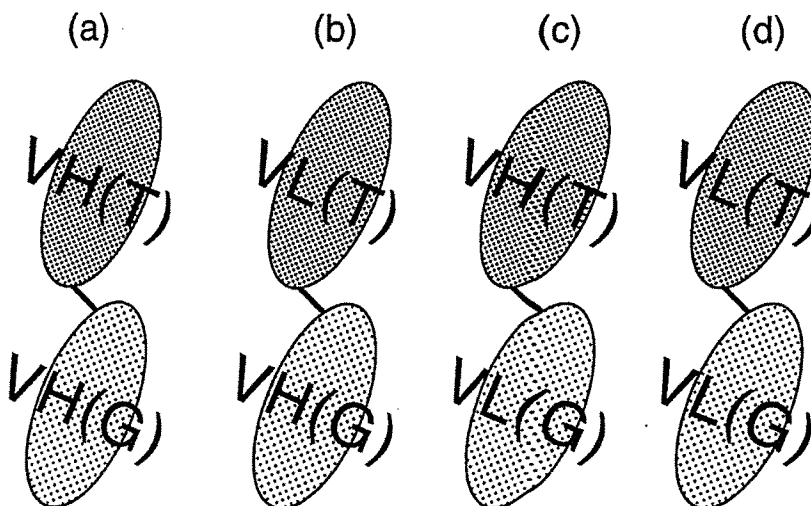
(57) Abstract: A protein utilizing an anti-gold antibody and a gold-binding side which is a part of the anti-gold antibody is constructed. This protein is capable of specifically binding to gold. This protein or a complex protein containing such a protein can be used for the detection of a target substance.

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DESCRIPTION

GOLD-BINDING PROTEIN AND USE THEREOF

5   TECHNICAL FIELD

The present invention relates to a gold-binding

protein, a complex protein containing the gold-

binding protein, and uses thereof for detecting their

target substances.

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BACKGROUND ART

Semiconductor industry has been developed

markedly by miniaturizing devices with an improvement

in recent semiconductor microprocessing technology.

15 The microprocessing technology typified by

lithography has recently achieved an accuracy of

finishing of several hundred nanometers. Materials

and devices applied with the above technology can

participate actively in many scenes and have been

20 expected to be applied in a wide variety of fields of

optical communication, electric communication, and so

on as well as of biotechnology and energy. However,

when consideration is given to processing on the

scale of 100 nanometers or less as an extension of

25 current microprocessing technology, there remain many

challenges for industrial use including the time and

expense involved in processing in addition to

technical challenges. With a growing number of
applicable fields, novel technology of manufacturing
refined structures as an alternative to the above has
been strongly demanded.

5   Under the circumstances, research and
development have been actively conducted on novel
materials by means of a bottom-up approach that
produces desired structures and properties controlled
at the atomic or molecular level, instead of

10   conventional top-down processing technology. An
example of bottom-up technology of producing refined
structures includes the development of a molecular
device in which a molecular arrangement is controlled
on a metal surface, and the use of methods of

15   utilizing the self-assembly of substances has been
studied as one of such technologies of molecular
arrangement control. As a recent example of the
study, Lindsay et al., (Science, 300: p. 1413, 2003)
has examined a molecular switching function by the

20   use of a self-assembled monolayer where alkanethiol
and alkanedithiol having thiol groups at their
respective molecular ends are oriented on a gold
substrate.

It has been known that biomolecules typified by
25   nucleic acids and proteins are constructed as precise
structures under control at the atomic level for
exerting their functions. The uses of the properties

of such biomolecules have been also under

investigation to apply the biomolecules to a variety

of devices in which the biomolecules are arranged on

a substrate of metal, metal oxide, or semiconductor.

5   Technology of producing a fine structure where the

biomolecule is arranged on a substrate becomes

important for a first step of developing such devices.

For example, when consideration is given to the

immobilization of a deoxyribonucleic acid (DNA) on a

10   gold substrate or the immobilization of a peptide

having an amino acid sequence with various functions

thereon, it has been widely known that the DNA or the

peptide can be chemically synthesized and the ends of

these substances are chemically modified with thiol

15   groups (-SH) to thereby coordinate these substances

on a gold substrate with the use of S-Au surface

absorption. Using this fact, study has been

conducted on experimental systems and conversion into

devices in which DNA or peptide is immobilized on

20   gold.

On the other hand, proteins that function as

enzymes, antibodies, or the like have high molecular

weights. Thus, it is very difficult to chemically

synthesize such compounds having high molecular

25   weights to form higher order structures while keeping

abilities to exert their functions. Although a

variety of studies have been currently conducted, the

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situation is that functional proteins that combine

their higher order structures with desired functions

have not been synthesized yet in most cases (Science,

302: p. 1364, 2003). When the functional protein is

5   immobilized on a substrate material, binding with the

substrate is generally performed by treating the

substrate with a variety of surface treatment agents

typified by a silane coupling agent, and introducing

into the protein a functional group capable of

10 binding to the surface of the substrate that was

subjected to surface treatment (Proteomics, 3: p. 254,

2003). However, it has been pointed out that such

introduction of a reactive functional group into the

functional protein is generally performed by chemical

15 modification and an introduction site is

nonselectively determined. Therefore, the functional

protein is immobilized in a less functionable shape

on the substrate and may be reduced in the resulting

activity due to the modification of the functional

20 expression site of the functional protein.

It is also possible to introduce a binding site

into a protein by a genetic engineering procedure to

produce a fusion protein. As an example, there is

known a method in which all sites or the biotin-

25 binding site of (strept) avidin which is known to

bind to a low molecular compound, biotine, is

introduced into the N- or C-terminal of a desired

protein by genetic engineering and the desired
protein is then expressed as a fusion protein that is
in turn immobilized via biotin arranged on the
surface of a desired substrate.

5   Furthermore, another technique has been
recently disclosed. In this case, a peptide composed
of five or more amino acids capable of binding to a
substrate material for immobilization. The peptide
may be fused to a desired protein to produce a fusion
10   protein, thereby binding and immobilizing the desired
protein on the substrate. Belcher et al., has
disclosed a peptide composed of 12 amino acids
capable of specifically recognizing a certain crystal
surface containing GaAs by the phage display method
15   (Nature, 405: p. 665, 2000), creating the new
possibilities for studying devices with the use of
the ability of biomaterials to self-assemble.
Moreover, Belcher et al., discloses the amino acid
sequence of a peptide composed of 7 to 14 amino acids
20   for other semiconductors (PbS, CdS) (WO 03/029431).
Brown et al., discloses some examples of a peptide
with a repetitive structure having the unit of an
amino acid sequence composed of 14 residues, showing
affinity for gold (Nature Biotechnology, 15: p. 269,
25   1997). Heretofore, a number of other peptides having
affinity for metals (Au, Pt, Pd, Ag), metal oxides
(SiO$_2$, ZnO, Cr$_2$O$_3$, Fe$_2$O$_3$), or semiconductors have been

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obtained by the above-described phage display method .

By immobilizing a desired biomolecule on a

substrate via such a peptide having affinity for the

substrate material, a structure cyclically having a

5   desired function and shape can be also constructed in

a self-assembly manner due to the interaction between

biomolecules or with a different substance. For

example, Belcher et al., discloses technique in which

M13 phage displaying ZnS-affinity peptide bound with

10   ZnS particles is coordinated by self-assembly to

thereby produce a liquid crystal-like film (Science,

296: 892, 2002).

In addition, some examples show a desired

functional protein to which a substrate-binding site

15   is fused is produced by genetic engineering as

described above to thereby allow the functional

protein to be immobilized at a desired site other

than the functional site on the substrate. However,

when peptide having affinity for a substrate material

20   is lined to the end of a functional protein directly

or via a linker composed of a few amino acids and

then bound to a solid phase (e.g., substrate) to

achieve the immobilization of the protein, the active

site of the functional protein (e.g., several amino

25   acid residues constituting the antigen-binding site

of an antibody) is close to the substrate and

undergoes some interaction (e.g., electrostatic

`effect) from the surface of the substrate to cause

structural changes and the like, resulting in a fear

of incapable of sufficiently exerting a desired

function. Even when a linker is designed to be still

5   longer, peptide as the linker, which has a high

degree of freedom in the motion of the molecules, may

be arranged close to a functional site and its

function may be inhibited.

From the above problems, the inventors of the

10   present invention have arrived at the idea that a

substrate-binding site on which functional polymeric

materials including a functional protein linker are

immobilized needs to have a structural portion

(scaffold) given as a spacer that keeps a certain

15   distance from a substrate without inhibiting desired

activity of an immobilized protein, and a site that

binds to the substrate.

As a molecule-recognizing molecule having such

a scaffold, the most well known is antibody. The

20   antibody is one of the proteins functioning in a self

defense mechanism to specifically recognize and bind

to a variety of structures on the surface of a

foreign substance invading animal's body fluid that

is subsequently detoxicated by the immune system.

25   The diversity of antibodies (the number of antibodies

having different amino acid sequences for binding to

a variety of foreign substances) is estimated to be

10^7 to 10^8 different varieties per animal. The structure is formed of polypeptide chains having two longer chains and two shorter chains and the longer polypeptide chain (heavy chain) and the shorter

5   polypeptide chain (light chain) are referred to as a heavy chain and a light chain, respectively.

Those heavy and light chains each have a variable region and a constant region. The light chain is a polypeptide chain composed of two domains

10   of one variable region (VL) and one constant region (CL), while the heavy chain is a polypeptide chain composed of four domains of one variable region (VH) and three constant regions (CH1-CH3). Each of the above-described domains assumes a cylindrical

15   structure composed of approximately 110 amino acids, and forms the layer structure of β sheets antiparallely arranged, and this layer structure is bound with one SS bond to form a highly stable structure. Moreover, it is known that the binding of

20   the antibody to a variety of antigen species results from the diversity of amino acid sequences in three complementarity determining regions (CDF) of each of the above-described variable regions (VH or VL). The CDRs, three for VH and three for VL, are separately

25   arranged by framework regions and recognizes the spatial configuration of functional groups in a recognition site of interest to allow highly specific

molecular recognition.

The diversity of the above-described CDR is attributed to DNA rearrangement that takes place in the antibody locus when a myeloid stem cell is

5   differentiated into a B lymphocyte as an antibody-producing cell. It is known that a portion composed of VH, D, and JH gene fragments in the heavy chain, and a portion composed of Vλ or Vκ and Jλ or Jκ gene fragments in the light chain undergo DNA

10   rearrangement to thereby produce antibodies. Such DNA rearrangement independently takes place in each B cell, one B cell produces only one kind of antibody. However, the whole B cells in an individual can produce diverse antibodies.

15   Antibody capable of binding to a specific substance as described above has been artificially produced heretofore using an antibody-forming mechanism in the immune system of an animal, and utilized in a variety of industrial fields. An

20   example of production methods is a method in which an animal to be immunized (e.g., rabbit, goat, or mouse) is immunized at constant intervals with an antigenic substance of interest along with an adjuvant, and antibodies present in its serum are collected. The

25   antibodies obtained as above are a mixture of several antibodies recognizing a variety of structures in the surface of the antigenic substance used in the

immunization. Serum containing several antibodies

binding to one antigen is referred to as a polyclonal

antibody.

On the other hand, a variety of B lymphocytes

5   that produce antibodies binding to an antigen of

interest are present in the spleen of an animal to be

immunized. Such an antibody-forming B lymphocyte is

fused to an established tumor cell to thereby allow

the production of a hybridoma cell. As described

10   above, one B lymphocyte produces one kind of antibody,

and a system that can subculture the B lymphocyte

producing one kind of antibody as a hybridoma has

been established. The antibody produced as above is

referred to as a monoclonal antibody.

15   As a molecular recognition structure using the

above-described structure of the antibody as an

anchorage, JP 05-055534 A discloses a multibinding

antibody recognizing two different antigens and a

method of forming a multilayer using the same.

20   According to this technique, a fusion antibody

recognizing a first antigen and a second antigen can

be obtained. In addition, the first antigen, the

fusion antigen, and the second antigen are

successively arranged on the surface of a substrate

25   to thereby allow the formation of a multilayer

without chemical modification. However, for

obtaining the disclosed fusion protein, it is

necessary to use an animal cell, turning into a problem in terms of cost efficiency and operational complication. Besides, when the multilayer is formed, a step of arranging an antibody recognizable by the

5   fusion protein on the surface of the substrate must be provided.

An antibody fragment, Fab, Fab', or F(ab')$_2$, obtained by treating the above-described antibody with a certain proteolytic enzyme, is known to have

10 the ability to bind to an antigen similar to that of the parent antibody.

Likewise, for the above-described VH, VL, or Fv as a complex thereof, or even for a complex composed of the VH or the VL, single chain Fv (scFv) having

15 the carboxy terminal of one region and the amino terminal of another region linked via peptide composed of several amino acids, or the like, is known to have the ability to bind to an antigen similar to that of the parent antibody.

20 Skerra and Better disclose a method in which Fab-type and Fv-type antibody fragments, the N terminals of which are added with a secretion signal sequence by a genetic engineering method, are produced as the expression of antibody genes by E.

25 coli (Science, 240: P. 1038, 1988; Science, 240: p. 1041, 1988). Alternatively, JP 07-501451 A discloses a multivalent antigen-binding antibody and a method

of producing the same, and JP 08-504100 A discloses a
multivalent and multibinding antibody and a method of
producing the same. Those two techniques disclose
that a binding protein is a protein containing an

5   antibody variable region portion (VH and/or VL)
binding to one or more antigens. JP 07-501451
discloses the structures and amino acid sequences of
binding proteins each recognizing pancarcinoma
antigen TAG-72 and fluorescein and a bispecific

10   antibody recognizing both, as well as base sequences
encoding them. JP 08-504100 A discloses, in Examples,
a divalent and bispecific binding protein composed of
an antibody fragment complex for known proteins (cell
membrane protein, cancer antigen CEA, FcR_Y1, etc.,)

15   or low molecular compounds.

However, in the above-described disclosures, no
technique concerning a binding protein that directly
recognizes and binds to the surface of a substrate
material typified by an inorganic substance is

20   disclosed. Therefore, when a structure in which the
binding protein is arranged on the substrate is
produced, the arrangement must be performed by a
conventionally known method, for example, a method
involving chemically modifying the substrate or the

25   above-described binding protein to arrange the
binding protein on the substrate through a covalent
bond. Similarly, when the binding protein is bound

to a fine particle of a metal or a semiconductor
substance, it is also necessary to chemically modify
the particle to be bound or binding protein. Such
chemical modification often targets amino residues or
5   carboxyl groups contained in large numbers in the
molecule of a protein, and a site involved in the
reaction is nonselective. Therefore, a site exerting
desired activity may be a substrate-binding or
labeling site and, as a result, the desired activity
10   of the protein may be reduced. Moreover, such a
problem is likely to arise in not only the technique
of conversion into microdevices but the production of
sensing elements such as biosensors. Thus, it is
important to immobilize a molecule capturing a target
15   substance such as an antibody on a substrate while
molecular orientation that sufficiently exerts its
capturing function is maintained.

Studies are being conducted, in which a protein
having a scaffold similar to that of the above-
20   described antibody is added with the ability to
recognize diverse molecules. Examples thereof
include anticolin (Review in Molecular Biotechnology,
74: p. 257, 2001) and a fibronectin type III domain
(J. Mol. Biol, 284: p. 1141, 1998). Anticolin is a
25   capture protein altered on the basis of lipocalin.
Lipocalin is a protein composed of 160 to 180 amino
residues, which functions in transporting and storing

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substances with a low degree of solubility in water.
Regarding constitution, lipocalin is a barrel
structure composed of 8 β sheets. Lipocalin can
recognize and bind to a substance of interest through

5   4 loop structures connecting the 8 β sheets.
Fibronectin is generally a protein composed of amino
acids not more than 100 residues, which plays a key
role in the junction of cells with extracellular
matrix or cell junction. Like the two proteins

10   described above, fibronectin is a protein that has β
sheet structures and recognizes a target substance
through loop structures among the β sheets. A novel
binding protein has been constructed by introducing
random amino acid sequences into the loop structures

15   among the β sheets of anticolin or fibronectin
described above. Because those molecules have a
strong molecular structure composed of β sheets in
addition to a molecular recognition site, the
molecules specifically recognize and bind to a

20   substrate by the fusion to a desired functional
polymeric material to thereby allow the polymeric
material to be immobilized on the substrate.
Moreover, the molecules are also expected to have a
spacer function that keeps a certain distance from

25   the substrate without inhibiting the desired activity
of the immobilized polymeric material. However,
there has been no reported case that molecule-

recognizing proteins with constitutively stable
scaffolds typified by the above-described antibody
specifically recognize and bind to inorganic
substances typified by metals or semiconductor
5   materials.

On the other hand, in the field of detection of
a variety of substances (target substances), several
methods of the detection and/or quantification and
the like of the target substances have been
10   established so far, particularly for proteins such as
antigens and antibodies, and sugar, lectin, and
nucleic acids. For example, it is known that a
labeling agent is bound to an antibody specifically
recognizing and binding to a sugar or a lectin as
15   target substances to thereby allow the detection or
quantification of the target substance via the
labeling agent. In general, a fine particle composed
of a metal such as gold or an organic material such
as latex, a fluorescent substance emitting
20   fluorescence by excitation light in a certain
wavelength region, or an enzyme having the
fluorescent substance as a reaction product (e.g.,
HRP) is used as a labeling agent. Methods of
labeling proteins such as antibodies include a method
25   by physical absorption and a chemical bond method in
which a reactive functional group is introduced into
a labeling agent or a substance to be labeled and is

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used as a crosslinking point to form a chemical bond.

Hereinafter, prior arts will be described by

taking antibodies used in detection as an example.

JP 03-108115 B discloses an example of a method of

5   labeling an antibody with gold by physically

absorbing a gold fine particle onto the antibody.

According to this method, a monoclonal antibody is

added to a dispersion of gold colloid that has been

previously adjusted, and the whole is subjected to

10   centrifugal sedimentation to remove a supernatant

liquid, and the resulting solution undergoes washing

processes several times to allow the production of an

antibody labeled with gold.

Next, a method of labeling antibodies by a

15   chemical bond method will be described. Antibodies

have the amino groups or SH groups of proteins. A

functional group reactive with those groups is

previously provided in a labeling agent to thereby

allow the chemical bond between a labeling agent such

20   as a fluorescent substance and a substance to be

labeled such as an antibody. An example of the

method includes a method involving introducing a

labeling agent having an N-hydroxysuccinimide group,

an isothiocyanate group, a nitroaryl halide group, or

25   an acid chloride group, which reacts with an amino

group. N-hydroxysuccinimide that is widely used as a

crosslinking agent for labeling proteins is known to

efficiently react with an amino group under a pH

atmosphere of 7 or more and form a highly stable

amide bond (Biochemistry, Vol. 11, pp. 2291, 1972).

The amino groups of the α position and of the ε

5   position of lysine side chain on a protein can be

targeted by a succinimide group in reaction. In

particular, an amino group at the ε position is

considered to be the general target of succinimide.

For example, when a gold fine particle as a labeling

10   agent is chemically bound to a protein such as an

antibody, the gold fine particle is first modified

with a compound having at least a SH group at one end

and a functional group highly reactive with the

above-described side chain residue of a protein at

15   another end. Next, the protein can be crosslinked

with the reactive functional group to bind them.

However, because a residue having lysine or a free α-

amino group nonselectively becomes a subject of

interest in reaction in those methods, a protein to

20   be labeled such as an antibody may be inhibited in

its function. Although FITC is also known as a

fluorescent substance having an isothiocyanate group,

like succinimide, the desired property of a protein

to be labeled may be reduced due to nonspecific

25   reaction to an amino group.

Alternatively, a -SH group can be given as a

crosslinking point. Methods with the use of a SH

group can be broadly divided into a maleimide method
and a pyridyl disulfide method. The maleimide method
is a method with the use of a crosslinking agent
having maleimide as a group selectively reactive with
5   a SH group, which is known to allow selective
crosslinkage under a mild condition. For example,
when a subject to be bound is an antibody, a SH group
that cleaved disulfide in the hinge portion of the
antibody molecule is unrelated to the antigen-
10   recognizing portion, so that the specificity of the
antibody is expected not to be impaired even though
the SH group is modified. This SH group is used as a
crosslinking point to thereby allow the binding of a
labeling agent without impairing a desired function.
15   However, an antibody has 16 SS bonds within the whole
molecule, including SS bonds for retaining the
structures of a heavy chain variable region (VH) and
a light chain variable region (VL) having a
complementarity determining region (CDR) as the
20   antigen-recognizing portion. Thus its function may
be impaired if the reduction of the SS bond is not
site-specifically performed.

JP 04-070320 B discloses a protein-labeling
technique with the use of metallothionein or a
25   fragment thereof, which is a low-molecular-weight
protein capable of chelation with high affinity for a
wide variety of metal ions. In this application, a

technique is disclosed, in which metallothionein

binds at the sulfhydryl moiety to a metal ion as a

labeling agent and is bound to an antibody or the

like at the other functional group given as a

5   crosslinking point such as an amine group, a hydroxyl

group, or a carboxyl group. Although sites binding

to a metal ion and to a substance to be labeled are

distinguished in metallothionein and can be bound

with the respective substances to be bound, the

10   binding site of the substance to be labeled is

uncertain as in other crosslinking methods and

problems as described above is likely to still remain.

Furthermore, means of resolving nonselective

modification due to the immobilization method on a

15   labeling agent through chemical crosslinking as

described above includes a modification method by

genetic engineering. It is also possible to produce

a fusion protein in which a binding site is

introduced into a protein by a genetic engineering

20   approach. Known as an example is a method in which a

low molecular compound biotin is chemically

introduced into the end of a labeling agent such as a

fluorescent substance, and the whole (strept) avidin

known to bind to the above-described biotin or a

25   biotin-binding site is introduced into the N terminal

or C terminal of a desired protein by genetic

engineering, which is in turn expressed as a fusion

protein and bound to the labeling agent via the
biotin-avidin bond. Thus, a low molecular compound
is introduced into a labeling agent such as a
fluorescent substance and a fusion protein in which a

5   protein capable recognizing and binding to the low
molecular compound is introduced into a desired
protein is produced by genetic engineering to allow
the introduction of a selective binding site.

However, the disclosed technique described

10   above does not disclose any technique concerning a
binding protein molecule-selectively recognizing and
binding to the surface of a substrate material
typified by an inorganic substance or a labeling
agent. Therefore, when a structure in which the

15   binding protein or a complex protein is arranged on a
substrate is produced, the arrangement must be
performed by a conventionally known method, for
example, a method involving chemically modifying the
substrate or the above-described binding protein to

20   arrange the binding protein on the substrate through
a covalent bond. Similarly, when the binding protein
is bound to a fine particle of a metal or a
semiconductor substance, and a labeling agent, it is
also necessary to chemically modify the fine particle

25   to be bound or the binding protein. Such chemical
modification often targets amino residues or carboxyl
groups contained in large numbers in the molecule of

a protein, and a site involved in the reaction is
nonselective. Therefore, a site exerting desired
activity may be a substrate-binding or labeling site
and, as a result, the desired activity of the protein
5   may be reduced.

Moreover, such a problem is likely to arise in
not only the technique of conversion into
microdevices but the production of sensing elements
such as biosensors. Thus, it is important to
10 immobilize a molecule capturing a target substance
such as an antibody on a substrate while molecular
orientation that sufficiently exerts its capturing
function is maintained.

15 DISCLOSURE OF THE INVENTION
A protein capable of binding to gold according
to the present invention is a protein having affinity
for gold, and is characterized by including a gold-
binding site containing at least a portion of an
20 antibody to gold.

According to one aspect of the present
invention, there is provided a gold-binding complex
protein, including:

(1) a first domain containing a protein having
25 affinity for gold; and

(2) a second domain containing a protein having
a binding site to a certain substance.

According to another aspect of the present
invention, there is provided a structure, including a
substrate and a protein, in which the substrate
contains gold in at least a portion of its surface

5   and the protein is a gold-binding complex protein
having the above configuration.

According to another aspect of the present
invention, there is provided a nucleic acid encoding
a gold-binding complex protein having the above

10 configuration. According to another aspect of the
present invention, there is provided a vector
including the nucleic acid.

According to another aspect of the present
invention, there is provided a method of producing a

15 structure having the above configuration, including
the step of:

    (1) preparing the substrate;

    (2) producing the gold-binding complex protein;
and

20   (3) arranging the gold-binding complex protein
on the substrate.

According to another aspect of the present
invention, there is provided a kit for detecting a
target substance, including a substrate and a gold-

25 binding complex protein for forming a structure
having the above configuration, and detection means
for detecting the binding of the target substance to

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the structure.

　　　According to another aspect of the present
invention, there is provided a connecting member for
labeling a target substance with a labeling agent,

5　including:

　　　one or more sites binding to the target
substance and one or more sites binding to the
labeling agent, in which:

　　　the site binding to the target substance and

10　the site binding to the labeling agent each
independently bind to a substance to be bound; and

　　　at least one of the site binding to the
labeling agent and the site binding the target
substance contains a protein having the above

15　configuration.

　　　According to another aspect of the present
invention, there is provided a method of detecting a
target substance, in which the target substance is
bound and labeled with a labeling agent to detect the

20　target substance bound with the labeling agent,
including the step of:

　　　binding the labeling agent to the target
substance via a connecting member having the above
configuration.

25　　　According to another aspect of the present
invention, there is provided a kit for detecting a
target substance, including: a labeling agent; a

connecting member having the above configuration; and
detection means for detecting a state in which the
labeling agent is bound to the target substance via
the connecting member.

5   The gold-binding complex protein according to
the present invention includes a gold-binding site
and a structure portion. Therefore, when a
functional substance linking and binding to the gold-
binding protein is immobilized on the surface of a
10   gold substrate, the immobilization does not affect
the original function of the functional substance.
Moreover, because no reagent is used in the
immobilization, the functional substance does not
suffer from any chemical reaction that affects its
15   function. Furthermore, by keeping a distance from
the gold substrate, the functional substance does not
undergo interaction from the substrate that affects
its function.

        In addition, the gold-binding complex protein
20   of the present invention includes several binding
sites to at least gold and a certain substance. This
allows a structure forming a layered body composed of
at least (i) a substrate having gold in at least a
portion of its surface, (ii) the gold-binding complex
25   protein of the present invention, and (iii) a certain
substance capable of binding to the gold-binding
complex protein of the present invention. In this

case, because the gold-binding complex protein of the
present invention has a sterically stable β sheet
structure of an antibody variable region domain,
space can be kept between the gold substrate and the
5   certain substance, and the domain (e. g., the second
domain) of the gold-binding protein, which is bounded
with gold or a substance other than gold (e.g., a
labeling agent) does not undergo some interaction
from the substrate containing gold and can retain the
10   binding ability. Moreover, this allows the formation
of a very thin and fine layered structure. Using
those properties of the gold-binding protein of the
present invention, a detection device can be obtained.
For example, the gold-binding complex protein of the
15   present invention capable of binding to a desired
substance is provided in a thin gold layer, a sensing
element for the desired substance can be made. A
detection method thereof can provide optical means,
for example, means with the use of surface plasmon
20   resonance.

On the other hand, when a substance containing
gold is given as a labeling agent, the gold-binding
protein of the present invention can be used as a
connecting member for binding the labeling agent
25   containing gold to a target substance. According to
this form as the connecting member, representative
effects described below can be obtained.

As a first effect, the gold-binding protein has
one or more of each of sites binding to a target
substance and a labeling agent and each of the
binding sites binds to a substance to be bound

5   independently from each other to thereby provide an
excellent connecting member capable of labeling the
target substance and not showing the reduction in the
ability of a substance to be labeled to bind to the
target substance, which is perceived as a problem

10   caused by the binding of the labeling agent in
conventional labeling method. As a second effect,
the connecting member is a biopolymer, more
particularly, a protein, and is thereby expected to
have high affinity resulting from the interaction of

15   the surface of the target substance with several
amino residues of the protein. As a third effect,
the connecting member is an antibody variable region
and is expected to have binding specificity because
the binding site is defined in the configuration

20   determined by the higher order structure. As a
fourth effect, the labeling agent is a substance
containing gold to thereby allow not only the
measurement of the quantity of scattered light for a
sample, but electric measurement with the use of its

25   electric properties, in addition to optical
measurement to which the principles of enhanced Raman
or localized plasmon are applied. As a fifth effect,

using the connecting member according to the pres ent

invention, a detection method and a detection kit

added simultaneously or optionally with the targe t

substance/connecting member/labeling agent can be

5   provided.

BRIEF DESCRIPTION OF THE DRAWINGS

    Fig. 1 is a diagram schematically showing the

constitution of a structure in an example of a

10   complex protein according to the present inventio n.

    Figs. 2A and 2B are, respectively, a diagram

schematically showing the constitution of a struc ture

in an example of a complex protein according to t he

present invention.

15   Fig. 3 is a diagram schematically showing the

constitution of a structure in an example of a

complex protein according to the present inventio n.

    FIGS. 4A and 4B are, respectively, a diagram

schematically showing the constitution of a struc ture

20   in an example of a complex protein according to t he

present invention.

    Fig. 5 is a diagram schematically showing the

constitution of a structure in an example of a

complex protein according to the present inventio n.

25   FIG. 6 is a diagram schematically showing the

constitution of a structure in an example of a

complex protein according to the present inventio n.

Fig. 7 is a diagram schematically showing the
constitution of a structure in an example of a
complex protein according to the present invention.

Fig. 8 is a diagram schematically showing the
5   constitution of a structure in an example of a
complex protein according to the present invention.

Fig. 9 is a diagram showing an example of the
SPR evaluation of VL obtained by the present
invention.

10   Fig. 10 is a diagram showing an example of the
SPR evaluation of VH obtained by the present
invention.

Fig. 11 is a schematic diagram of a vector for
illustrating Examples of the present invention.

15   Figs. 12A and 12B are schematic diagrams of a
vector for illustrating Examples of the present
invention.

Fig. 13 is a schematic diagram of a vector for
illustrating Examples of the present invention.

20   Fig. 14 is a schematic diagram of a vector for
illustrating Examples of the present invention.

FIG. 15 is a schematic diagram of a vector for
illustrating Examples of the present invention.

Fig. 16 is a schematic diagram of a vector for
25   illustrating Examples. of the present invention.

Fig. 17 is a diagram showing an example of the
SPR evaluation of scFv obtained by the present

invention.

Fig. 18 is a SPR chart in Example 21.

Fig. 19 is a GPC chart in Example 31.

Fig. 20 is a SPR chart in Example 32.

5   Fig. 21 is a SPR chart in Example 34.

Fig. 22 is a SPR chart in Example 36.

Fig. 23 is a GPC chart in Example 37.

Fig. 24 is a SPR chart in Example 38.

Fig. 25 is a GPC chart in Example 40.

10   Fig. 26 is an absorption curve of a solution

containing a gold fine particle in Example 42.

Fig. 27 is a SPR chart in Comparative Example 3.

Fig. 28 is a SPR chart in comparative Example 4.

15   BEST MODES FOR CARRYING OUT THE INVENTION

Hereinafter, a gold-binding protein according

to the present invention, a complex protein by the

use of the same, and uses thereof will be described.

(1) gold-binding protein

20   Antibody

The gold-binding protein of the present

invention includes at least a portion of an antibody.

Examples of an antibody used for the binding with

gold as shown in the present invention includes

25   antibodies having an affinity for gold that are

produced by lymphoid cells in all vertebrates, and a

protein having an amino acid sequence with the

30

deletion, substitution, or addition of one or several

amino acids in the amino acid sequence of the

antibodies and having relationship with the

antibodies in the structure and the function, that is,

5　maintaining a desired affinity for gold. Although

antibodies are categorized according to the

classification of their properties (immunological or

physical) into IgG, IgM, IgA, IgD, and IgE, any of

antibodies in the classification may be used in the

10　present invention. Moreover, those antibodies may

form a multimer. For example, IgA forms a dimer and

IgM forms a pentamer, presenting no problem as long

as they are in a shape capable of binding to gold.

Procurement of gold-binding antibody. Moreover,

15　antibodies may be IgW and IgY as long as they are for

in vitro use.

As a method of obtaining the gold-binding

antibody of the present invention, a technique of

preparing anti-serum and a technique of producing a

20　monoclonal antibody by cell fusion, which have been

conventionally performed, can be appropriately

selected and performed. For example, an appropriate

animal for immunization is immunized with a gold fine

particle to be bound in the present invention, and an

25　antibody is collected from serum after the

confirmation of increase in the antibody titer. The

above-described immunization is generally performed

by a method in which a gold fine particle as an
antigen is diluted to an appropriate concentration
with an appropriate solvent, for example, a
physiological saline solution, and this solution is
5   intravenously or intraperitoneally administered
together with, if necessary, the Freund complete
adjuvant, to an animal 3 to 4 times at intervals of 1
to 2 weeks. The animal thus immunized is anatomized
at the 3 day post-final immunization and a splenocyte
10   obtained from the excised spleen is used as an
immunocyte. An immunizing gold fine particle has a
size of preferably 10 nm or smaller, more preferably
2 nm or smaller. In addition, it is more preferred
that the gold fine particle be covalently attached to
15   a protein such as serum albumin and be rendered
hapten. Heretofore, it has been expected that an
antigen hard to produce immune reaction is also
recognized as a portion of a certain protein antigen
and its production is induced by the formation of a
20   complex between the antigen and the protein antigen.

The obtained antibody may be polyclonal and is,
however, given as a monoclonal antibody to thereby
allow the selection of a clone having high
specificity for gold. The monoclonal antibody can be
25   obtained by cloning a cell producing it. In general,
an immunoglobulin-forming cell such as the splenocyte
collected from an immunized animal is fused to a

32

tumor cell to thereby allow the formation of a

hybridoma (Gulfre G., Nature 266. 550-552, 1977).

For example, tumor cells include myeloma cells such

as a mouse-derived myeloma P3/X63-AG8. 653 (ATCC No.

5   CRL-1580), P3/NSI/1-Ag4-1 (NS-1), P3/X63-Ag8. U1

(P3U1), SP2/0-Ag14 (Sp2/O, Sp2), NS0, PAI, FO, or

BW5147, a rat-derived myeloma 210RCY3-Ag. 2. 3., and

a human-derived myeloma U-266AR1, GM1500-6TG-A1-2,

UC729-6, CEM-AGR, DIR11, or CEM-T15.

10   In the screening of a cell producing the

monoclonal antibody, the cell is cultured in a titer

plate and the reactivity of a culture supernatant in

a well where proliferation is observed to the above-

described gold fine particle can be measured by, for

15   example, enzyme immunoassay such as radio immunoassay

(RIA) or enzyme-linked immuno-solvent assay (ELISA),

or immunoprecipitation. Alternatively, affinity for

gold can be also quantitatively measured with a

surface plasmon resonance (SPR) device.

20   Antibody fragment

An antibody fragment described in the present

invention refers to the region of a portion of a

monoclonal antibody, and specific examples thereof

include F(ab')2, Fab', Fab, Fd, a variable fragment

25   of antibody (Fv), single chain Fv (scFv), disulphide

stabilized Fv (dsFv), and a single domain antibody

(dAv) composed of a heavy chain variable region (VH)

or a light chain variable region (VL).

"F(ab')2" and "Fab'" as used herein can be obtained by treating an antibody with, for example, pepsin or papain as a proteolytic enzyme. They refer

5   to antibody fragments produced by digestion in the vicinity of a disulfide bond present between two heavy chains (H chains) in a hinge region of an antibody. For example, when IgG is treated with papain, the IgG is cleaved upstream of a disulfide

10   bond present between two H chains in the hinge region to give two homologous fragments in which a light chain (L chain) composed of a light chain variable region (VL) and a light chain constant region (CL) and a heavy chain (H chain) composed of a heavy chain

15   variable region (VH) and a heavy chain constant region 1 (CH1) are bound through a disulfide bond at the C-terminal region. Each of those two homologous antibody fragments is referred to as Fab'. Alternatively, when IgG is treated with pepsin, the

20   IgG is cleaved downstream of a disulfide bond present between two H chains in the hinge region to allow the production of an antibody fragment slightly larger than the above-described two Fab' linked in the hinge region. This antibody fragment is referred to as

25   F(ab')2.

The gold-binding protein of the present invention may be Fab' or F(ab')2 described above.

The gold-binding protein may also be an Fd fragment
in which VH and the above-described CH1 are bound.

Moreover, the gold-binding protein may be a
variable fragment of antibody (Fv) or a portion

5   thereof, for example, a heavy chain variable region
(VH) or a light chain variable region (VL)
constituting Fv or a portion thereof. On the other
hand, for a complex composed of the above-described
VH or VL, a single chain Fv (scFv) having the carboxy

10 terminal of one region and the amino terminal of
another region linked via peptide composed of several
amino acids can be used. It is preferred that VH/VL
(not in particular order) formed of the above-
described scFv be provided with a linker composed of

15 one or more amino acids. It is important that the
residue length of the amino acid linker be designed
not to have such a binding force that inhibits the
formation of a structure necessary for the binding of
the antigen with VH or VL. As a specific example,

20 the amino acid linker generally has 5 to 18 resides
in length, with 15 residues more widely used and
studied. Those fragments can be obtained by a
genetic engineering approach.

Moreover, although either of VH and VL may be

25 single domain dAb, the single domain structure is
often unstable in general and may be therefore
stabilized by chemical modification such as PEG

modification. Moreover, variable region, VHH of
camelid heavy chains (J. Mol. Biol, 311: p123, 2001)
and variable region of immunoglobulin-like molecules
of Nurse Shark, IgNAR that exist and can function as
5   heavy chain only antibodies in vivo can be used.
Moreover, interface of VH/VL or alike may be
introduced by drawing upon equivalents of heavy chain
only antibodies to increase stability when VH or VL
of antibdies that are composed of heavy chain and
10 light chain represented by those of humans and mice
are used as domain units as shown in Figs. 1 to 4.

      A gold-binding site can include at least one
selected from (1) antibody heavy chain variable
region (VH), a variant, and a portion thereof, and
15 (2) antibody light chain variable region (VL), a
variant, and a portion thereof. Preferable examples
of the antibody heavy chain variable region (VH)
include a protein including at least one of amino
acid sequences of SEQ ID NOs.: 1 to 48, and
20 preferable examples of the antibody light chain
variable region (VL) include a protein including at
least one of amino acid sequences of SEQ ID NOs.: 49
to 57. A protein having affinity for gold and
containing one or more amino acid sequences with the
25 deletion, substitution, or addition of one or several
amino acids in each of these amino acid sequences of
SEQ ID NOs.: 1 to 57 can be similarly utilized.

Procurement of antibody fragment having affinity for
gold
Procurement by enzyme treatment

5   The above-described antibody is treated with
some enzyme to allow the procurement of an antibody
fragment having the antigen-binding site and the
antigen-binding ability of the antibody to some
extent. For example, a Fab fragment or an analogue

10   thereof can be obtained by treating the obtained
antibody with papain. A F(ab')$_2$ fragment or an
analogue thereof can be obtained by treating the
obtained antibody with pepsin. The above-described
antibody fragment is also produced by a chemical

15   degradation method in addition to the above-described
enzymatic approach. This antibody fragment can be
used without a problem as long as it has the ability
to bind to gold.

      A method of obtaining the above-described Fab',

20   Fv, or dAb of VH or VL according to the present
invention may also be procurement by the use of a
genetic engineering approach. An example thereof is
a method in which a gene library of the VH or VL is
produced and comprehensively expressed as a protein

25   to select a corresponding gene based on affinity for
gold or a target substance. The gene library can be
obtained from, for example, cord blood, tonsil, bone

marrow, or peripheral blood cells or splenocytes.
For example, mRNA is extracted from a human
peripheral blood cell and cDNA is synthesized. Next,
a sequence encoding human VH or VL is used as a probe
5   to produce a cDNA library of the human VH or VL. For
example, primers capable of extensively amplifying
human VH family (VH1 to 7) per family and primers
capable of amplifying human VL are known in the art.
RT-PCR is performed by combining a primer with each
10   of such VH or VL to give a gene encoding the VH or VL.
Alternatively, it is also possible to use a phage
display method. In the phage display method, a gene
library encoding VH, VL, or a complex containing them
(e.g., Fab, scFv) is bound to a gene encoding a coat
15   protein of the phage to produce a phagemid library
that is in turn transformed into E. coli and
expressed as phages having a variety of VHs or VLs as
a portion of the coat protein. Those phages are used
to allow selection based on affinity for gold or a
20   target substance, as described above. A gene
encoding VH or VL that is displayed as a fusion
protein by the phage is encoded by the phagemid in
the phage and can be therefore identified by DNA
sequencing.
25   The present invention also includes a nucleic
acid encoding the above-described gold-binding
protein. The present invention further includes a

constituent composed of a nucleic acid that is given

as a gene vector to be expressed as a protein for

transforming a host cell (e.g., a conventionally

known protein-expressing cell derived from E. coli.,

5   yeasts, mice, or humans) to allow the expression of

the above-described gold-binding protein. The gold-

binding protein of the present invention that can be

expressed by one expression vector can be selected

and designed from the whole molecules of an antibody

10   or an antibody fragment thereof F(ab')2, Fab,

Fv(scFV), VH, or VL, or a complex thereof. When a

plurality of the above-described antibody fragments

are encoded by one expression vector, each of the

antibody fragments can be independently expressed as

15   an individual polypeptide chain. It is also possible

to construct a vector that expresses the antibody

fragments as one polypeptide chain in which the

domains are linked successively or via an amino acid.

In the constitution of the vector for

20   expressing the gold-binding protein of the present

invention, the vector can be designed and constructed

by integration into constitution or the like

necessary for expressing a transgene, such as a known

promoter, depending on a selected host cell. The

25   vector can be constructed with reference to the

constitution of a known promoter or the like

depending on a selected host cell. When E. coli or

the like is used as a host cell, the gold-binding
protein of the present invention or a constituent
thereof as a foreign gene product is immediately
removed into the outside of the cytoplasm to thereby

5   allow reduction in degradation by protease. Moreover,
it is known that, even if this foreign gene product
is toxic for a bacterium, its effect can be reduced
by secreting the foreign gene product to the outside
of the bacterium. Most proteins secreted through a

10   known cytoplasmic membrane or inner membrane usually
have signal peptides at the N terminals of their
precursors, which are cleaved by signal peptidase in
the secretion process to turn the precursors into
mature proteins. Most signal peptides have basic

15   amino acids, hydrophobic amino acids, and cleavage
sites by signal peptidase, which are located at their
N terminals.

The gold-binding protein of the present
invention can be secreted and expressed by locating a

20   nucleic acid encoding a conventionally known signal
peptide typified by pelB on the 5' side of a nucleic
acid encoding the gold-binding protein.

Several gold-binding proteins of the present
invention or several polypeptide chains each composed

25   of plural antibody fragments can be also inserted
each independently into one vector. In this case, a
nucleic acid encoding pelB can be located on the 5'

side of each domain or a nucleic acid encoding the

polypeptide chain to facilitate secretion.

Alternatively, when the gold-binding protein is

expressed as a polypeptide chain composed of one or

5   more domains, a nucleic acid encoding pelB can be

located on the 5' side of the polypeptide chain to

thereby facilitate secretion, as described above.

The gold-binding protein of the present invention in

which a signal peptide is fused at its N terminal as

10   described above can be purified from a periplasm

fraction and a medium supernatant.

In consideration of convenience in procedures

of purifying the expressed protein, it is possible to

arrange, by genetic engineering, a tag for

15   purification at the N or C terminal of a polypeptide

chain formed of an antibody molecule, or each

independent antibody fragment or several antibody

fragments successively linked. Examples of the

above-described tag for purification include a

20   histidine tag composed of six consecutive histidine

residues (hereinafter, HisX6) and the glutathione-

binding site of glutathione-S-transferase. Examples

of a method of introducing the tag include: a method

in which a nucleic acid encoding the tag for

25   purification is inserted at the 5' or 3' end of a

nucleic acid encoding the gold-binding protein in the

above-described expression vector; and the use of a

commercially available vector for introducing the tag
for purification.

A method of producing the gold-binding protein
of the present invention by the use of the above-
described expression vector will be described
hereinafter. A conventionally known host protein-
expressing cell is transformed with a vector for
expressing the gold-binding protein, which is
designed in accordance with the host cell, to
synthesize the gold-binding protein of the present
invention or the polypeptide chain as a component
thereof into the host cell by the use of a protein
synthesis system in the host cell. Thereafter, a
desired protein accumulated or secreted outside or
inside the host cell can be purified and thereby
obtained from the inside of the cell or a cell
culture supernatant. For example, when E. coli is
used as a host cell, the E. coli can be constructed
to facilitate secretion and expression to the outside
of the cytoplasm by locating a nucleic acid encoding
a conventionally known signal peptide typified by
pelB on the 5' side of a nucleic acid encoding the
gold-binding protein of the present invention.

When several polypeptide chains constituting
the gold-binding protein of the present invention are
expressed in one expression vector of the present
invention, a nucleic acid encoding pelB can be

located on the 5' side of a nucleic acid encoding

each of the polypeptide chains to facilitate

secretion to the outside of the cytoplasm at its

expression. The gold-binding protein of the present

5   invention in which a signal peptide is fused at its N

terminal as described above can be purified from a

periplasm fraction and a medium supernatant. In a

purification method, a nickel chelate column can be

used for purification when the tag for purification

10   is a His-tag, and an immobilized glutathione column

can be used for purification when the tag for

purification is GST.

     The gold-binding protein of the present

invention that is expressed in a bacterium can be

15   also obtained with insoluble granules. In this case,

the insoluble granules can be centrifuged from a cell

homogenized solution in which the bacterium obtained

from culture solution is homogenized with a French

press or ultrasound. The obtained insoluble granule

20   fraction can be solubilized with a buffer solution

containing a conventionally known denaturant having

urea and guanidine hydrochloride, followed by

purification with a column as described above under a

denaturation condition. For the obtained column

25   elution fraction, the removal of the denaturant and

the reconstruction of an active structure can be

performed by a refolding procedure. As the refolding

procedure, any of conventionally known methods can be
appropriately used. More particularly, a stepwise
dialysis method or dilution method can be used
depending on a desired protein.

5   Each of the domains or each of the polypeptide
chains of the gold-binding protein of the present
invention can be expressed in an identical cell or
can be complexed coexistently with one another after
being expressed using another host cell.

10   In addition, using a vector encoding the gold-
binding protein of the present invention, the protein
can be also expressed in vitro with a cell extract
solution. Examples of the cell extract solution
preferably used include E. coli, a wheat germ, and a

15 rabbit reticulocyte. However, the synthesis of a
protein with the cell extract solution is generally
performed under a reduction condition. Therefore, it
is more preferred that some treatment be performed
for forming a disulfide bond in an antibody fragment.

20   The preferred dissociation constant (K_D) of the
gold-binding protein of the present invention is 10^{-6}
M or less under a buffer condition in the presence of
0.1% Tween 20, more preferably 10^{-8} M or less. The
inventors of the present invention have confirmed by

25 study that a protein material cannot attach to gold
on the above-described condition even for the protein
material such as BSA having relatively large

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nonspecific attachment to gold. If the K_D value is
10^{-6} or less, the attachment behavior of a
nonspecifically adhesive protein as described above
can be sufficiently distinguished. In addition, when
5 the K_D value is 10^{-8} or less, the gold-binding protein
can sufficiently function as an anchor molecule for
immobilization.

Expression of antibody fragment protein

   The gold-binding protein is cleaved with a
10 desired restriction enzyme, for example, NcoI/NheI in
the above-described case, to give DNA encoding a
gold-binding antibody fragment. This can be
introduced into a conventionally known plasmid for
expressing a protein in accordance with a host cell
15 to thereby give an antibody fragment. For example,
when E. coli is used as a host cell and a desired
antibody fragment is collected from an extracellular
expression or periplasm fraction, a conventionally
known signal peptide can be introduced upstream of a
20 gene encoding the above-described antibody fragment.
The signal peptide includes pelB. For easily
purifying a desired protein from a culture
supernatant or a bacterial fraction after expression,
a conventionally known tag for purification may be
25 fused. More particularly, six histidine residues
(HisX6) or the glutathione-binding site of
glutathione-S-transferase can be introduced to give a

fusion protein. In the case of the His-tag, this
fusion protein can be easily purified with a metal
chelate column such as nickel. In the case of the
GST tag, purification can be performed with a column
5   having a carrier such as Sepharose on which
glutathione is immobilized.

    Alternatively, when a desired protein expressed
in a bacterium cannot be obtained with insoluble
granules, the insoluble granules can be solubilized
10   with a conventionally known buffer solution
containing urea and guanidine hydrochloride, followed
by refolding. Any of conventionally known methods
can be appropriately used as the refolding procedure.
More particularly, a stepwise dialysis method or
15   dilution method can be used depending on a desired
protein.

    An antibody obtained as above and a fragment
thereof, for example, Fab, (Fab')2, Fc, VH or VL, or
a complex thereof even having the deletion,
20   substitution, or addition of one or several amino
acids in their amino acid sequences do not depart
from the scope of the present invention as long as
they have affinity for gold.

    The preferred dissociation constant (K_D) of the
25   gold-binding protein of the present invention is 10^{-6}
M or less under a buffer condition in the presence of
0.1% Tween 20, more preferably 10^{-8} M or less. The

inventors of the present invention have confirmed by
study that a protein material cannot attach to gold
under the above-described condition even for the
protein material such as BSA having relatively large
5   nonspecific attachment to gold.

If the K_D value is 10^{-6} or less, the attachment
behavior of a nonspecifically adhesive protein can be
sufficiently distinguished. In addition, when the K_D
value is 10^{-8} or less, the gold-binding protein can
10   sufficiently function as an anchor molecule for
immobilization.

Substance to be bound having gold

A substance to be bound having gold can be
15   selected and used from various substances having gold
in at least a portion of their surfaces. When the
gold-binding protein is selected by immunization,
panning, or the like, the surface of the substance to
be bound is composed of only gold for eliminating the
20   contamination of proteins attaching to substances
other than gold. Materials for the inner core
substrate other than the surface can be selected and
used from, as a matter of course, gold, and a variety
of the other known materials. Moreover, the
25   substance to be bound is preferably given in a
particulate form, more preferably as a fine particle
having 1 to 100 μmφ, to thereby allow increase in the

specific surface area involved in the binding and to
allow easy collection by centrifugation for
collection at the completion of panning. In addition,
the substance to be bound may be one obtained by
5   evaporating gold onto any one of a variety of
commercially available plastic plates, for example, a
culture dish or a 96-well titer plate, as described
below. In this case, in consideration of the wetted
area of the gold surface and the molecular diffusion
10 effect by stirring, the size and the number of wells
are preferably determined.

The shape of the substance to be bound can be
appropriately selected and used from shapes known in
the art such as flattened, spherical, acicular, and
15 porous shapes. A formation method can be
appropriately selected from a physical or chemical
vapor deposition method or a chemical production
method with the use of gold chloride. The obtained
surface containing gold may be previously washed with
20 an acidic solution, an alkaline solution, an organic
solvent, or the like, in order to eliminate
impurities such as oxide layers, by-products, and
contaminants and have desired condition of gold
exposure.
25 Substrate

A structure available in a variety of uses can
be obtained from a gold-binding protein and a

substrate having gold forming at least a portion of
its surface. A substrate having any shape and
material can be used as long as it is provided with
gold in at least a portion of its surface and can

5   form the structure of the present invention. The
material of the substrate used in the present
invention may be any of materials that can form the
structure of the present invention, and that include
any one or more or a complex thereof selected from a

10   metal, a metal oxide, an inorganic semiconductor, an
organic semiconductor, glasses, ceramics, a natural
polymer, a synthetic polymer, and a plastic. The
shape of the substrate used in the present invention
may be any of shapes that can form the structure of

15   the present invention, and that include any one or
more selected from plate, particulate, porous body-
like, protruded, fibrillary, cylindrical, and
reticular shapes.

      Examples of the organic polymer compound for

20   forming a substrate include an organic polymer
compound manufactured through polymerization of
polymerizable monomers selected from the group
consisting of: styrene polymerizable monomers such as
styrene, α-methylstyrene, β-methylstyrene, o-

25   methylstyrene, m-methylstyrene, p-methylstyrene, 2,4-
dimethylstyrene, p-n-butylstyrene, p-tert-
butylstyrene, p-n-hexylstyrene, p-n-octylstyrene, p-

n-nonylstyrene, p-n-decylstyrene, p-n-dodecylstyrene,
p-methoxystyrene, and p-phenylstyrene; acrylate
polymerizable monomers such as methyl acrylate, ethyl
acrylate, n-propyl acrylate, isopropyl acrylate, n-
5 butyl acrylate, isobutyl acrylate, tert-butyl
acrylate, n-amyl acrylate, n-hexyl acrylate, 2-
ethylhexyl acrylate, n-octyl acrylate, n-nonyl
acrylate, cyclohexyl acrylate, benzyl acrylate,
dimethyl phosphate ethyl acrylate, diethyl phosphate
10 ethyl acrylate, dibutyl phosphate ethyl acrylate, and
2-benzoyloxy ethyl acrylate; methacrylate
polymerizable monomers such as methyl methacrylate,
ethyl methacrylate, n-propyl methacrylate, isopropyl
methacrylate, n-butyl methacrylate, isobutyl
15 methacrylate, tert-butyl methacrylate, n-amyl
methacrylate, n-hexyl methacrylate, 2-ethylhexyl
methacrylate, n-octyl methacrylate, n-nonyl
methacrylate, diethyl phosphate ethyl methacrylate,
and dibutyl phosphate ethyl methacrylate; methylene
20 aliphatic monocarboxylic acid esters; vinyl esters
such as vinyl acetate, vinyl propionate, vinyl
benzoate, vinyl butyrate, vinyl benzoate, and vinyl
formate; vinyl ethers such as methyl vinyl ether,
ethyl vinyl ether, and isobutyl vinyl ether; and
25 vinyl polymerizable monomers including vinyl ketones
such as methyl vinyl ketone, hexyl vinyl ketone, and
isopropyl vinyl ketone.

Further, examples of the inorganic solids may

include, but are of course not limited to: clay

minerals such as kaolinite, bentonite, talc, and

mica; metallic oxides such as alumina, titanium

5   dioxide, zinc oxide, magnetite, ferrite, NbTa

composite oxide, WO_3, In_2O_3, MoO_3, V_2O_5, and SnO_2;

insoluble inorganic salts such as silica gel,

hydroxyapatite, and calcium phosphate gel; metals

such as gold, silver, platinum, and copper;

10   semiconductor compounds such as GaAs, GaP, ZnS, CdS,

and CdSe; glass; silicon; and composites thereof.

Examples of films and sheets that may be used

for forming the substrate of the present invention

include, but are of course not limited to: films

15   composed of plastics such as polyethylene

terephthalate (PET), diacetate, triacetate,

cellophane, celluloid, polycarbonate, polyimide,

polyvinyl chloride, polyvinylidene chloride,

polyacrylate, polyethylene, polypropylene, and

20   polyester; porous polymer membranes composed of

polyvinyl chloride, polyvinyl alcohol, acetyl

cellulose, polycarbonate, nylon, polypropylene,

polyethylene, and teflon; wooden boards; glass

boards; silicon substrates; fabrics such as, cotton,

25   rayon, acrylic, silk, and polyester; paper such as

good quality paper, medium quality paper, art paper,

bond paper, recycled paper, baryta paper, cast coated

paper, corrugated paper, and resin coated paper. Note
that the materials of those films and sheets may also
be smooth or uneven.

Examples of the substrate include: base plates

5   made of silicon, silica, glass, quartz glass, and so
on, microflow paths and holes (pores) formed in the
base plates by means of photolithography, etching,
and sand blast, or those having surfaces on which
thin films of gold, silver, and platinum are formed;

10 base plates made of polydimethyl siloxane (PDMS),
polymethyl methacrylate (PMMA), polyethylene
terephthalate (PET), polycarbonate (PC), polystyrene
(PS), and so on and microflow paths and holes (pores)
formed therein by means of molding techniques; carbon

15 nanotubes, carbon nanohones, fullerenes, diamonds, or
aggregates thereof; nanowhiskers constructed of
alumina, carbon, flullerene, ZnO, and so on;
mesoporous thin films, fine particles, and monolith
structural members made of SiO_2, aluminosilicate,

20 other methallosillicates, TiO_2, SnO_2, Ta_2O_5, and so
on; fine particles of gold, silver, copper, platinum,
and so on; iron-oxide fine particles of magnetite,
ferrite, hematite, gammna hematite, maghemite, and so
on; an aluminum/silicon mixture films and silicon-

25 oxide nano structural members obtained therefrom by
means of anodic oxidation; porous alumina thin films;
alumina nonohole structures; and silicon nano-wires,

but not limited to them.

In addition, the dimensions of the substrate of
the present invention can be selected variously
depending on its intended use application.

5   · Target Substance Detecting Kit

A kit for detecting a target substance can be
obtained using the configuration of the gold-binding
protein of the present invention added with affinity
to the target substance. For instance, a kit for

10   detecting a target substance can be constructed of:
both a substrate and a gold-binding complex protein
for forming the above structural member; and a
detecting member for detecting the binding of a
target substance to the structural member. For

15   instance, the binding of a protein containing a gold-
binding protein to a target substance can be detected
such that gold or a labeling agent containing gold is
added to a target substance in a state of being bound
to a protein containing a gold-binding protein and a

20   physical or chemical procedure is then carried out to
detect the gold or the target substance.
Alternatively, when a target substance is one that
contains gold, the affinity of a gold-binding protein
to gold is utilized to allow the gold-binding protein

25   to bind to the gold-containing labeling agent and the
binding state thereof is then detected. In this case,
the marker used may be one which will be described

later in the description of a connecting member.
Furthermore, for example, the binding between the
labeling agent and the gold-binding protein can be
detected by means of a change in physical value, such

5   as an optical, electrical, or thermal change.

By the way, the structure added with the
affinity to the labeling agent may be preferably one
that utilizes a gold-binding complex protein
described later or a gold-binding protein as a

10 binding member between a target substance and a
labeling agent.
·Surface Plasmon Resonance Apparatus

In addition, the binding of a protein that
contains a gold-binding protein to gold can be

15 quantitatively determined using, for example, a
conventionally-known surface plasmon resonance
measuring apparatus. In general, the surface plasmon
resonance is a method of determining a change in
refractive index on a gold thin film formed on a

20 glass base plate by a change in resonance angle
generated by resonance between free electrons on the
gold thin film and an evanescent wave generated on
the boundary surface between glass and gold by
incident light from a glass side at an incident angle

25 of not more than the total reflection angle. The
determined change in refractive index can be
converted into the amount of a subject protein to

gold and then evaluated.

· Dissociation Constant (K_D)

The term "dissociation constant (K_D)" refers to
a value obtained by dividing a "binding rate (Ka)" by

5   a "dissociation rate(kd)" value. Those rates may be
used as indexes that represent the affinity of the
monoclonal antibody or a fragment thereof to gold.
The rates can be analyzed according to any of various
methods. In the present invention, however, the

10   rates are determined from a binding curve using a
measuring device, Biacore2000 (Amersham Pharmacia
Biotech, Co., Ltd.) according to an analysis software
bundled on the device.

(2) Gold-binding complex protein

15   The gold-binding complex protein of the present
invention includes two or more domains. Of those
domains, at least one domain contains the gold-
binding protein as constructed above. Exemplified
complex proteins include those constructed as

20   described below.

(a) A complex protein including a first domain
1 including a gold-binding protein as constructed
above and a second domain 2 including a protein
having a binding site specific to a particular

25   substance.

(b) A complex protein further including at
least one of a third domain 3 that forms a complex

with the first domain and a fourth domain 4 that
forms a complex with the second domain in addition to
the first domain and the second domain.

In addition, at least one of the second to
5   fourth domains may contain at least one protein
having affinity for gold. In this case, each of
those domains can be constructed by incorporating the
gold-binding protein constructed as described above.
Furthermore, the first to fourth domains may have
10 affinity to a subject binding substance and their
affinity can be then adjusted independently from one
domain to another. Alternatively, two or more
domains may have similar affinity. Those domains may
be constructed from domains having different affinity.
15   Furthermore, at least two selected from the
first to fourth domains may be contained in the same
polypeptide chain. Examples of such a configuration
of the gold-binding complex protein include the
following structures.
20   (1) The structure in which the first domain and
the second domain form one polypeptide chain.
(2) The structure in which the first domain and
the second domain are bound via one or more amino
acids.
25   (3) The structure in which the third domain and
the fourth domain form one polypeptide chain.
(4) The structure in which the third domain and

the fourth domain are bound via one or more amino
acids.

    (5) The structure consisting of a first
polypeptide chain containing a first domain and a
second domain and a second polypeptide chain
containing a third domain and a fourth domain.

    (6) The structure consisting of a first
polypeptide chain containing a first domain and a
second domain and a third polypeptide chain
containing a third domain and a second domain.

    (7) The structure consisting of a first
polypeptide chain containing a first domain and a
second domain and a fourth polypeptide chain
containing a first domain and a fourth domain.

    (8) The structure consisting of one polypeptide
chain containing at least a first domain, a second
domain, and a third domain.

    (9) The structure consisting of one polypeptide
chain containing at least a first domain, a second
domain, and a fourth domain.

    (10) The structure consisting of one
polypeptide chain containing first to fourth domains.

· Gold-binding Complex Protein

    A protein provided as a constituent of the
gold-binding complex protein refers to a molecule
that contains at least one polypeptide chain formed
by allowing at least two amino acids to bind together,

where the polypeptide chains can be folded into a
specific three-dimensional structure to exert its
inherent functions (e.g., conversion and molecular
recognition). In addition, the gold-binding complex
5   protein of the present invention is a complex protein
that contains at least one binding site for gold and
also at least one binding site for gold or other
substance except gold to show affinity with
polyvalent or multiple specificity. For instance,
10   the complex protein may preferably include a first
domain having a binding site for gold and containing
at least a part of the variable region of a light
chain (VL) or the variable region of a heavy chain
(VH) of an antibody and a second domain having a
15   binding site for a specific substance (hereinafter,
referred to as a target substance) and containing at
least a part of VH or VL. Hereinafter, VH and VL
binding with gold refer to VH(G) and VL(G),
respectively. VH and VL binding with a target
20   substance refer to VH(T) and VL(T), respectively.

The antibody heavy chain variable region (VH)
and the antibody light chain variable region (VL) are
variable regions which respectively belong to an
antibody heavy chain and an antibody light chain. In
25   general, each of the antibody heavy chain variable
region (VH) and the antibody light chain variable
region (VL) is of a tubular structure and consists of

about 110 amino acids and a layered structure is then
formed with β sheet groups being arranged in opposite
directions, while the layer structure is connected
using a single S-S bond to form a very stable

5   structure. In addition, it is known that the
variable region (VH or VL) is a complementarity
determining region (CDR) that determines the binding
of antibodies to a wide variety of antigens. There
are three CDRs in each of VH and VL and are separated

10   from each other by framework regions having amino
acid sequences with comparatively low diversity to
thereby recognize a spatial arrangement of a
functional group of a target recognition site,
allowing the more advanced recognition of a specific

15   molecule.

Hereinafter, an example of the gold-binding
complex protein of the present invention will be
described. As schematically illustrated in Fig. 1,
the minimum unit of the gold-binding complex protein

20   includes the first domain and the second domain.
Exemplified combinations of the domains include
VH(G)-VH(T) as shown in (a), VH(G)-VL(T) as shown in
(b), VL(G)-VH(T) as shown in (c), and VL(G)-VL(T) as
shown in (d). In this example, the first domain

25   binds to gold and the second domain binds to a target
substance, independently, without forming a
complementary binding site between the first and

second domains.   The first and second domains may be

independent polypeptide chains, respectively, or they

may be continuously aligned and linked with each

other.   In terms of simplification of the

5   manufacturing process and functional expression, a

more preferable embodiment is to form a polypeptide

chain by continuously linking the polypeptide chains.

In the case of the polypeptide chain prepared by

continuously linking the first domain and the second

10   domain, the first domain and the second domain may be

directly linked with each other or may be linked

through a linker 5 made of one or more amino acids.

The linker consisting of amino acids is preferably

one consisting of 1 to 10 amino acids, more

15   preferably one consisting of 1 to 5 amino acids.

When the linker has an amino acid length of 11 to 15,

complementary binding between the first domain and

the second domain (becoming scFv) may be formed

because of little restriction imposed on those

20   domains due to their arrangements. For preventing

the formation of a complementary complex between VH

and VL, it is known that it is effective to burden

the structural limitation between the domains by

shortening the linker length. It is desirable that

25   structural change that is brought when each of the

first and the second domains binds to target

substance does not effect its desired binding ability

to target substance. Therefore, it is possible to set
the linkers comprised of second structure such as
□⁻helix or insert polypeptide that is not related to
required binding character, as long as they do not
5   significantly effect desired characters or
productivity.

Furthermore, the gold-binding complex protein
of the present invention forms a complex with the
first domain. It may include a third domain that
10   contains at least a part of VH or VL and/or a fourth
domain that contains at least a part of VL or VH
which forms a complex with the second domain. It is
more desirable to allow the third domain to make a
complex with the first domain to form a complementary
15   gold-binding site with the first domain.

For instance, when the first domain is VH(G) as
shown in the schematic diagrams in Figs. 2A and 2B,
the third domain is preferably VL which is able to
form FV with the first domain, more preferably a
20   structure formed such that a gold-binding site is
formed by linking the first domain with the third
domain.

As described above, the first domain and the
third domain form Fv to attain structural stability
25   and to be expected to prevent a decrease in function
due to the structural change. Furthermore, as the
third domain forms a gold-binding site by linking

with the first domain, a further increase in binding

ability (e.g., an increase in binding rate and

suppression of dissociation rate) can be expected.

Furthermore, as shown in Fig. 2A, the first

5   domain and the third domain may be respectively

provided as independent polypeptide chains or may be

linked with each other to form a polypeptide chain

(e.g., third domain - first domain - second domain,

as shown in the schematic diagram of Fig. 2B, the

10   structure may be suitably determined such that the

binding ability is exerted on gold and a target).

Furthermore, as another example, the structure as

shown in a schematic diagram of Fig. 3 may be

allowable. In other words, it is a complex

15   constructed of a polypeptide chain consisting of the

first domain and the second domain and a polypeptide

chain consisting of the third domain and the second

domain. In this case, the binding with gold is

attained by Fv or an Fv-like complex formed from the

20   first and third domains and the first domain then

functions as an anchor that binds to a target

substance through the second domain.

Furthermore, the gold-binding protein of the

present invention may include a fourth domain

25   consisting of at least. a part of VH or VL to form a

complex with the second domain. It is desirable to

form a binding site to the target substance together

with the second domain in a complementary manner.
For example, as shown in the schematic diagram of Fig.
4A, when the second domain is VL, the fourth domain
is preferably VH which is able to form Fv with the
5   second domain. More preferably, the second domain
and the fourth domain are linked together to form a
binding site against the target substance.
Furthermore, as shown in a schematic diagram of Fig.
4B, a polypeptide chain may be formed by linking the
10 first, second, and fourth domains together.
    Especially in case that the protein binds to
gold substrate via at least a portion of surface of
the first domain while binds to target substance via
the second and the fourth domain and that
15 irreversible structural change occurs when the first
domain binds to the substrate, it is possible to
minimize the effect on binding abilities of the
second and the forth domain by setting a linker.
    Moreover, the gold-binding protein of the
20 present invention may be constructed as illustrated
in a schematic diagram of Fig. 5. That is, it is a
complex constructed of a polypeptide chain consisting
of the first domain and the second domain and a
polypeptide chain consisting of the first domain and
25 the fourth domain. In this case, the complex binds
to a target substance by means of Fv or an Fv-like
complex which is made up of the second domain and the

'fourth domain to allow the first domain to function
as an anchor for combining both polypeptide chains
described above with gold.

Furthermore, the gold-binding protein of the
5   present invention may include the third and fourth
domains together as constituent materials. As shown
in a schematic diagram of Fig. 6, the third and
fourth domains may be independent polypeptide chains,
respectively. Alternatively, as shown in a schematic
10   diagram of Fig. 7, it may be a polypeptide chain
obtained by a linkage between polypeptide chains.
When the gold-binding protein is provided as a linked
polypeptide chain, the third domain and the fourth
domain may be directly linked together or may be
15   linked through a linker consisting of one or more
amino acids as shown in Fig. 7. The length of the
linker may be defined such that, as described above,
the linker consisting of amino acid(s) has an amino
acid length of preferably 1 to 10 amino acid, more
20   preferably 1 to 5 amino acids.

Furthermore, as shown in a schematic diagram of
Fig. 8, the first to fourth domains may be linked
together in a single polypeptide chain. In this case,
those domains are arranged such that the first domain
25   and the third domain can form a complex and then the
complex can bind to gold while the second domain and
the fourth domain can form a complex and then the

complex can bind to gold and other substance except
gold. Therefore, the linker is preferably provided
between the domains. For instance, 1 to 5 amino
acids are located between the first and second

5   domains or between the third and fourth domains.
Also, 15 to 25 amino acids are located between the
second and fourth domains of amino acids. In the
similar structure, each of or both of the first and
second domains and the third and fourth domains may

10   be replaced with each other.

The sequence of each domain in the single
polypeptide chain may be suitably selected and
defined depending on desired properties such as
affinity for gold or a target and the long-term

15   stability of the gold-binding protein.

The first domain is, for example, one
comprising at least one amino acid sequence selected
from SEQ ID No: 1 to SEQ ID No: 57. In each of those
amino acid sequences, there is no problem as far as

20   the amino acid retains its affinity for gold even
though the amino acid sequence is subjected to the
deletion, substitution, or addition of one or several
amino acids. Furthermore, it may be used as the gold
binding protein of the present invention without any

25   problem as far as it shows affinity for gold even
though the amino acid sequence is provided as one
forming part of the amino acid sequences or provided

as a complex thereof. Specific examples of VH of the
present invention having SEQ ID No: 1 to SEQ ID No:
57 are represented in SEQ ID No: 58 to SEQ ID No: 74.
Also, specific examples of VL of the present
5   invention are represented in SEQ ID No: 75 to SEQ ID
No: 77.

    Preferably, the third domain contains one or
more amino acids selected from SEQ ID No: 1 to SEQ ID
No: 57. Furthermore, the third domain is more
10   preferably defined by suitably selecting from SEQ ID
No: 58 to SEQ ID No: 77 depending on the first domain.

    The present invention also includes a nucleic
acid that encodes the above gold-binding complex
protein. In addition, the present invention also
15   includes a construct consisting of a nucleic acid to
be provided as a vector for transforming host cells
(e.g., conventionally known protein-expressing cells
from E. coli, yeast, mouse, human, or the like).
Exemplified nucleic acid sequences that encode the
20   first and third domains capable of constituting the
gold-binding protein of the present invention are
represented in SEQ ID No: 98 to SEQ ID No: 116.

    Each domain of the gold-binding protein of the
present invention, which can be expressed by one
25   expression vector described above, may be designed by
selecting from 1 to 4. When one expression vector is
encoded by multiple domains of the gold-binding

protein of the present invention, each domain can be
expressed as an independent polypeptide chain. In
addition, a vector configuration expressed as one
polypeptide in which domains are bound directly or

5   via an amino acid can be obtained. Furthermore, the
configuration of the expression vector for the gold-
binding protein of the present invention can be
designed and constructed by incorporating transgenes
such as known promoters into a constitution required

10   for the expression thereof on the basis of host cells
selected. The configuration of the vector can be
constructed with reference to the configuration of
known promoters or the like on the basis of host
cells selected. Furthermore, when E. coli or the

15   like is used as host cells, the gold-binding protein
of the present invention, which is a foreign gene
product, is quickly removed out of cytoplasm to
reduce the decomposition of protease. Furthermore,
when the gene product may be toxic for bacterial

20   cells, it is possible to reduce its effect by
secretion to the outside of cell bodies. In general,
most of proteins secreted through the known cell
membrane or inner membrane have signal peptides on
their N terminals of the peptidase. During the

25   secretion step, the signal peptidase cleaves the
protein to make it a mature protein. Many signal
peptides have basic amino acids, hydrophobic amino

acids, and the cleavage sites of the signal peptidase
at their N terminals.

    The peptide chain can be expressed and secreted
by arranging nucleic acid that encodes a

5   conventionally known signal peptide typified by a
single peptide pelB on the 5' end of the nucleic acid
encoding the gold-binding complex protein of the
present invention. In addition, each of domains that
constitute the gold-binding complex protein of the

10 present invention or a polypeptide chain constructed
of two or more of the domains may be independently
inserted into a single vector. In this case, the
nucleic acid that encodes pelB is arranged on the 5'
end of nucleic acid that encodes each of domains or a

15 polypeptide chain to facilitate the secretion. For
expressing the polypeptide chain as one consisting of
one or more domains, likewise, the secretion of such
a polypeptide chain can be facilitated by arranging
the nucleic acid that encodes pelB on the 5' end of

20 the polypeptide chain. In this manner, the gold-
binding protein of the present invention, in which a
signal peptide is fused on the N-terminal end thereof,
or a domain used as a constituent thereof can be
purified from the periplasma fraction and the

25 supernatant of the culture. In addition, considering
the easiness of work at the time of purifying the
protein expressed, a purification tag may be arranged

68

on each of independent domains or on the N or C

terminal of the polypeptide chain formed by combining

two or more domains by a genetic engineering

procedure. The purification tag may be a histidine

5   tag constructed of continuously arranged six

histidine residues (hereinafter, referred to as· His x

6), a glutathione-binding site of glutathione-S-

transferase, or the like. As a method of introducing

the tag, for example, there is a method in which a

10   nucleic acid encoding a purified tag is inserted into

the 5' or 3' terminal of nucleic acid encoding a

gold-binding protein in the expression vector, or a

method with the used of a commercially available

vector for introducing a purified tag.

15   Hereinafter, the method of manufacturing the

gold-binding complex protein of the present invention

using the above expression vector will be described.

The gold-binding complex protein of the present

invention or a polypeptide chain to be provided as a

20   constituent thereof is synthesized by: transforming a

vector for expressing the gold-binding complex

protein designed depending on host cells into host

cells for expressing a conventionally known protein;

and preparing a desired protein in the host cells

25   using a protein synthesis system in host cells.

After that, the intended protein being accumulated or

secreted inside or outside the host cells is purified

from the inside of the cells or the supernatant of

cell culture.

For instance, in the case of using E. coli as

host cells, nucleic acid that encodes a

5   conventionally known signal peptide typified by pelB

is arranged on the 5' end of nucleic acid that

encodes the gold-binding complex protein of the

present invention to thereby facilitate the secretory

expression. For expressing two or more polypeptide

10   chains to obtain each of domains that constitute the

gold-binding complex protein of the present invention

by means of one expression vector, nucleic acid that

encodes pelB is arranged on the 5' end of nucleic

acid that encodes each polypeptide chain to

15   facilitate the secretion to the outside of cytoplasm.

In this manner, the gold-binding protein of the

present invention, in which a single peptide is fused

on the N-terminal end thereof, can be purified from

the periplasma fraction and the supernatant of the

20   culture. As a purification method, when the

purification tag is a His tag and when a column is a

nickel chelate column or GST, the purification can be

carried out using a glutathione immobilization column.

Furthermore, In addition, the gold-binding

25   complex protein of the present invention expressed in

bacterial cells can be obtained in the form of

insoluble granule. In this case, the bacterial cells

obtained from a culture medium may be crushed by a
French press or an ultrasonic wave and then the
insoluble granules are centrifuged from a cell
homogenized solution. The resulting insoluble

5   granule fraction is dissolved in a buffer solution
containing a conventionally known denaturing agent
including urea and guanicine hydrochloride salt and
then purified through a column under denaturing
conditions as described above. The resulting column

10   eluent fraction may be subjected to a refolding work
to remove the denaturing agent and reconstruct an
active structure. The refolding method used may be
suitably selected from any of those known in the art.
Specifically, depending on a protein of interest, a

15   serial dilution method, a dilution method, or the
like may be used.

        Each of domains or each of polypeptide chains
of the gold-binding protein of the present invention
may be expressed in the same host cell and then

20   complexed, or alternatively expressed using other
host cells and allowed to coexist to make a complex.

        Furthermore, using a vector encoding the gold-
binding protein of the present invention, the in
vitro expression of a protein from a cell extract may

25   be carried out. The cell extracts appropriately used
herein include E. coli, wheat germ, and rabbit
reticulocytes. However, the protein synthesis in the

above cell-free extract is typically carried out under the reduction condition. Therefore, more preferable is to carry out any treatment for the formation of a disulfide bond in the antibody

5 fragment..

The gold-binding complex protein of the present invention has a dissociation constant (K_D) of 10^{-6} M or less, more preferably 10^{-8} M or less under the buffer condition of in the presence of 0.1% Tween 20.

10 Under the above conditions, the inventors of the present invention have studied and confirmed that none of protein materials which have been considered to show comparatively high adsorption to gold can be adsorbed to gold. When the Kd value is 10^{-6} M or less,

15 it can be sufficiently distinguishable from the adsorption behavior of protein having non-specific adsorptivity. Besides, 10^{-8} M or less allows a sufficient function as an anchor molecule for immobilization.

20 As one of the methods of acquiring an antibody heavy chain variable region (VH) or antibody light chain variable region (VL) having binding ability to a target substance having gold of the present invention, there is a method including: preparing a

25 gene library of VH or VL described above; expressing them as proteins cyclopaedically; and making correspondence between the proteins and the genes to

select one on the basis of affinity for gold or a
target substance. The above gene library can be
obtained from cord blood, tonsil, marrow, peripheral
blood cells, splenic cells, or the like. For

5   instance, mRNA is extracted from human peripheral
blood cells to prepare cDNA. Subsequently, using a
human VH- or VL-encoding sequence as a probe, cDNA
library of human VH or VL is prepared. For instance,
primers which can widely amplify the human VH

10 families (VH1 to 7) every family and primers which
can amplify human VL are known in the art. Every VH
or CL, primers are combined together to carry out RT-
PCR to acquire the VH- or VL-encoding gene.
Alternatively, a phage display method may be used.

15 The phage display method includes: combining a gene
library for encoding VH, VL, or a complex (e.g., Fab,
scFv) containing VH or VL with a gene that encodes a
phage coat protein; preparing phagemid libraries;
transforming the phagemid libraries into E. coli; and

20 expressing as phages having various VH or VL as part
of the coat protein. Using those phages, as well as
the above description, it can be selected depending
on affinity for gold or a target substance. Genes
encoding VH or VL represented as a fusion protein in

25 the phage is encoded in a phagemid of the phage.
Therefore, it can be specified by subjecting to a DNA
sequence analysis.

Furthermore, cells that produce an antibody of
interest are collected from an animal immunized with
gold or a target substance and then a VH or VL base
sequence or an amino acid sequence can be specified
5   using the same primers as those described above.

Furthermore, the third and fourth domains of
the present invention can be designed against a
target substance on the basis of an amino acid
sequence of a variable region in a known antibody or
10   a fragment thereof. In addition, when the antibody
or the fragment thereof against a target substance is
not obtained, it is possible to design as described
above by analyzing an amino acid sequence after
acquiring an antibody thereto. Both of the third
15   domain and the fourth domain may be those that
constitute the gold-binding protein of the present
invention. In this way, the VH or VL base sequence
thus obtained is used to prepare the gold-binding
complex protein of the present invention.
20   · Binding Object having Gold

As a binding object used for the selection of a
gold-binding protein by means of immunization,
panning, or the like, those previously exemplified in
the case of the gold-binding protein are available.
25   · Substrate

The gold-binding complex protein of the present
invention may be combined with a substrate to provide

a structure which can be used in various kinds of
applications. The substrates which can be used in
the applications are those previously exemplified for
the gold-binding protein.

5   · Target Substance

        The gold-binding complex protein of the present
invention is constructed of a domain having affinity
for gold and a domain having affinity for a target
substance and thus it becomes possible to utilize the
10   protein as a complex protein for detecting the target
substance. The target substance provided as a
detection subject may be any of molecules which can
be provided as antigens in the respective procedures
using antigen/antibody reactions. For instance, the
15   target substances can be roughly classified into non-
biological substances and biological substances.

        Examples of non-biological materials with a
high value of industrial utilization include PCBs and
dioxins that are environmental pollutants having
20   different chlorine substitution numbers and positions,
and endocrine-disrupting substances, so called
environment hormones (for example, hexachlorobenzene,
pentachlorophenol, 2,4,5-trichloroacetic acid, 2,4-
dichlorophenoxyacetic acid, amitrole, atrazine,
25   alachlor, hexachlorocyclohexane, ethylparathion,
chlordane, oxychlordane, nanochlor, 1,2-dibromo-3-
chloropropane, DDT, kelthane, aldrin, endrin,

dieldrin, endosulfan (benzoepin), heptachlor,
heptachlor epoxide, malathion, methomyl, methoxychlor,
mirex, nitrofen, toxaphene, trifluralin, alkylphenol
(with carbon numbers of 5 to 9), nonylphenol,

5   octynonylphenol, 4-octylphenol, bisphenol A, di-2-
ethylhexl phthalate, butylbenzyl phthalate, di-n-
butyl phthalate, dicyclohexyl phthalate, diethyl
phthalate, benzo(a)pyrene, 2,4-dichlorophenol, di-2-
ethylhexyl adipate, benzophenone, 4-nitrotoluene,

10   octachlorostyrene, aldicarb, benomyl, kepone
(chlordecone), manzeb (mancozeb), maneb, metiram,
metribuzin, cypermethrin, esfenvalerate, fenvalerate,
permethrin, vinclozolin, zineb, ziram, dipentyl
phthalate, dihexyl phthalate, and dipropyl phthalate).

15   The biological substances include a biological
substance selected from a nucleic acid, a protein, a
sugar chain, a lipid, and a complex thereof, and more
particularly include a biological substance selected
from a nucleic acid, a protein, a sugar chain, and a

20   lipid. In particular, the present invention can be
applied to any of substances containing a substance
selected from any of DNA, RNA, an aptamer, a gene, a
chromosome, a cell membrane, a virus, an antigen, an
antibody, lectin, hapten, a hormone, a receptor, an

25   enzyme, peptide, sphingoglycolipid, and sphingolipid.
In addition, a bacterium or cell itself that produces
the above-described "biological substance" can also

be a target substance as a "biological substance" of interest in the present invention.

A specific example of a protein includes a so-called disease marker. Examples thereof include: α-fetoprotein (AFP) as a marker for hepatocellular carcinoma (primary liver cancer), hepatoblastoma, metastatic liver cancer, and yolk sac tumor, which is an acidic glycoprotein produced by a hepatic cell in the fetal period and present in fetal blood; PIVKA-II that is an abnormal prothrombin appearing during hepatic parenchyma disorder and found to appear specifically for hepatocellular carcinoma; BCA225 as a marker for primary advanced breast cancer, and recurrent and metastatic breast cancer, which is a glycoprotein of an antigen immunohistochemically specific for breast cancer; basic fetoprotein (BFP) as a marker for ovarian cancer, testicular tumor, prostatic cancer, pancreatic carcinoma, biliary tract cancer, hepatocellular carcinoma, renal cancer, lung cancer, gastric cancer, bladder carcinoma, and colon cancer, which is a basic fetal protein found in a human fetal serum, intestine, and brain cell extract; CA15-3 as a marker for progressive breast cancer, recurrent breast cancer, primary breast cancer, and ovarian cancer, which is a sugar chain antigen; CA19-9 as a marker for pancreatic carcinoma, biliary tract cancer, gastric cancer, liver cancer, colon cancer,

and ovarian cancer, which is a sugar chain antigen;
CA72-4 as a marker for ovarian cancer, breast cancer,
clorectal cancer, gastric cancer, and pancreatic
carcinoma, which is a sugar chain antigen; CA125 as a

5   marker for ovarian cancer (especially, serous
cystadenocarcinoma), corpus uteri adenocarcinoma,
Fallopian tube cancer, uterocervical adenocarcinoma,
pancreatic carcinoma, lung cancer, and colon cancer,
which is a sugar chain antigen; CA130 as a marker for

10   epithelial ovarian cancer, Fallopian tube cancer,
lung cancer, hepatocellular carcinoma, and pancreatic
carcinoma, which is a glycoprotein; CA602 as a marker
for ovarian cancer (especially, serous
cystadenocarcinoma), corpus uteri adenocarcinoma, and

15   uterocervical adenocarcinoma, which is a core protein
antigen; CA54/61 (CA546) as a marker for ovarian
cancer (especially, mucinous cystadenocarcinoma),
corpus uteri adenocarcinoma, and uterocervical
adenocarcinoma, which is a mother cell nucleus sugar

20   chain-associated antigen; a carcinoembryonic antigen
(CEA) most widely used at present for assisting in
cancer diagnostic as a tumor-associated marker
antigen such as colon cancer, gastric cancer, rectal
cancer, biliary tract cancer, pancreatic carcinoma,

25   lung cancer, breast cancer, uterine cancer, and
urinary system carcinoma; DUPAN-2 as a marker for
pancreatic carcinoma, biliary tract cancer,

hepatocellular carcinoma, gastric cancer, ovarian

cancer, and colon cancer, which is a sugar chain

antigen; elastase 1 as a marker for pancreatic

carcinoma, pancreatic cisterna, and biliary tract

5   cancer, which is an exocrine pancreatic proteolytic

enzyme present in pancreas and specifically

hydrolyzing elastic fiber elastin of connective

tissues (constituting arterial wall, tendon, or the

like); an immunosuppressive acidic protein (IAP) as a

10   marker for lung cancer, leukemia, esophageal cancer,

pancreatic carcinoma, ovarian cancer, renal cancer,

cholangioma, gastric cancer, bladder carcinoma, colon

cancer, thyroid carcinoma, and malignant lymphoma,

which is a glycoprotein present at high

15   concentrations in ascites or serum of human cancer

patients; NCC-ST-439 as a marker for pancreatic

carcinoma, biliary tract cancer, breast cancer, colon

cancer, hepatocellular carcinoma, pulmonary

adenocarcinoma, and gastric cancer, which is a sugar

20   chain antigen; γ-seminoprotein (γ-Sm) as a marker for

prostatic cancer, which is a glycoprotein; a

prostate-specific antigen (PSA) that is a

glycoprotein extracted from human prostate tissues

and present in only prostate tissues, thereby being a

25   marker for a prostatic cancer; prostatic acid

phosphatase (PAP) used as a tumor marker for

prostatic cancer, which is an enzyme hydrolyzing

phosphate under acid pH secreted by prostate gland;

neuron-specific enolase (NSE) as a marker for lung

cancer (especially, lung small cell carcinoma),

neuroblastoma, nervous system neoplasm, pancreatic

5   islet cancer, Gullet small cell carcinoma, gastric

cancer, renal cancer, and breast cancer, which is a

glycolytic enzyme specifically present in nervous

tissues and neuroendocrine cells; a squamous cell

carcinoma-associated antigen (SCC antigen) as a

10   marker for uterine cancer (cervical squamous cancer),

lung cancer, esophageal cancer, Head and neck cancer,

and carcinoma cutaneum, which is a protein extracted

and purified from the liver metastatic focus of

uterocervical squamous cancer; a sialyl Le^{x}-i antigen

15   (SLX) as a marker for pulmonary adenocarcinoma,

esophageal cancer, gastric cancer, colon cancer,

rectal cancer, pancreatic carcinoma, ovarian cancer,

and uterine cancer, which is a sugar chain antigen;

SPan-1 as a marker for pancreatic carcinoma, biliary

20   tract cancer, liver cancer, gastric cancer, and colon

cancer, which is a sugar chain antigen; a tissue

polypeptide antigen (TPA) as a marker for esophageal

cancer, gastric cancer, colorectal cancer, breast

cancer, hepatocellular carcinoma, biliary tract

25   cancer, pancreatic carcinoma, lung cancer, and

uterine cancer, which is single-stranded polypeptide

useful especially in the estimation of progressive

cancer and recurrence precognition/therapeutic

process observation in combination with other tumor

markers; a sialyl Tn antigen (STN) as a marker for

ovarian cancer, metastatic ovarian cancer, gastric

5   cancer, colon cancer, biliary system cancer,

pancreatic carcinoma, and lung cancer, which is a

mother cell nucleus carbohydrate antigen; cytokeratin

(CYFRA) as an effective tumor marker for detecting

pulmonary non-small cell carcinoma, especially

10   pulmonary squamous cancer; pepsinogen (PG) as a

marker for gastric ulcer (especially, infraversion

gastric ulcer), duodenal ulcer (especially, recurrent

and intractable cases), Brunner's gland oma, Dzo

ringer Ellison syndrome, and acute gastritis, which

15   is an inactive precursor of two pepsins (PG I, PG II)

as a proteolytic enzyme secreted into gastric juice;

C-reactive protein (CRP) that is an acute phase

reactant changed in blood plasma by tissue injury and

infection and shows high level when necrosis occurs

20   in heart muscle due to acute myocardial infarction or

the like; serum amyloid A protein (SAA) that is an

acute phase reactant changed in blood plasma by

tissue injury and infection; myoglobin as a marker

for acute myocardial infarction, muscular dystrophy,

25   polymyositis, and dermatomyositis; which is a heme

protein having a molecular weight of approximately

17,500 and present mainly in heart muscle and

skeletal muscle; creatine kinase (CK)(three isozymes
of CK-MM type derived from skeletal muscle, CK-BB
type derived from brain and smooth muscle, and CK-MB
type derived from heart muscle, and mitochondrial

5   isozymes or immunoglobulin-bound type CK (macro CK))
as a marker for acute myocardial infarction,
hypothyroidism, progressive muscular dystrophy, and
polymyositis, which is an enzyme present mainly in
the soluble fraction of skeletal muscle or heart

10   muscle and emigrating into blood due to cellular
lesion; troponin T as a marker for rhabdomyolysis,
myocarditis, myocardial infarction, and renal failure,
which is a protein with a molecular weight of 39,000
forming a troponin complex with troponins I and C on

15   the thin filament of striated muscle and involved in
the regulation of muscular contraction; ventricular
muscle myosin light chain I as a marker for acute
myocardinal infarction, muscular dystrophy, and renal
failure, which is a protein contained in both

20   skeletal muscle and heart muscle and for which a rise
in the measurement means the disorder or necrosis of
skeletal muscle and heart muscle; or chromogranin A,
thioredoxin, and 8-OhdG that recently receive
increasing attention as a stress marker.

25 Kit for detecting target substance

        The gold-binding complex protein according to
the present invention can be used to constitute a kit

for detecting a target substance. For example, the
kit for detecting a target substance can be
constituted, which includes: a gold-binding complex
protein with the use of an antibody and a variant

5   thereof specifically binding to the target substance
in a second domain and a fourth domain optionally
used, a substrate having a surface containing gold;
and detection means for detecting the target
substance immobilized on the substrate via the gold-

10   binding complex protein. The detection of the target
substance immobilized on the gold substrate via the
gold-binding complex protein can be measured with the
above-described surface plasmon resonance device. A
method in which the substrate containing gold in a

15   potion thereof is used as a labeling agent to detect
the target substance can be utilized. The gold-
binding complex protein described above can be used
as a substance for mediating such a gold substrate,
labeling agent, and target substance. The gold-

20   binding complex protein formed using an antibody
fragment recognizing and binding to the target
substance can be used in the present invention.

     EXAMPLES

25   Hereinafter, the acquisition and evaluation of
a protein capable of binding to gold as an example of
the present invention will be described in detail.

However, the present invention is not limited to the contents described in "EXAMPLES".

Example 1 (Preparation of antibody heavy chain variable region VH library)

5   By using a Fab library derived from human adult peripheral blood B lymphocyte as a template, VH coding gene was subjected to DNA replication by means of the following primer in accordance with a method recommended by PCR (TAKARA BIO INC, LA kit). The

10   primer was set as follows.

·back primers

(SEQ ID No: 78)

5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCT GG-3'

15   (SEQ ID No: 79)

5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCCAGRTYCAGCTGGTGCAGTCT GG-3'

(SEQ ID No: 80)

5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCCAGSTRCAGCTGCAGSAGTCR

20   GG-3'

(SEQ ID No: 81)

5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCSARGTGCAGKTGGTGGAGTCT GG-3'

(SEQ ID No: 82)

25   5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCCCAGTGTGAGGTGCAGCTGGT GG-3'

(SEQ ID No: 83)

5'-TCGCAACTGCGGCCCAGCCGG<u>CCATGG</u>CCCAGGTGCAGCTACAGSAGTGG

GG-3'

·forward primers

(SEQ ID No: 84)

5   5'-ATTCTCGACTGCTAGCTGAGGAGACGGTGACCAGGGTGCC-3'

(SEQ ID No: 85)

5'-ATTCTCGACTGCTAGCTGAAGAGACGGTGACCATTGTCCC-3'

(SEQ ID No: 86)

5'-ATTCTCGACTGCTAGCTGAGGAGACGGTGACCAGGGTTCC-3'

10 . (SEQ ID No: 87)

5'-ATTCTCGACTGCTAGCTGAGGAGACGGTGACCGTGGTCCC-3'

        (1) By using the Fab library as a template, the

VH coding gene was amplified by PCR using the primer.

PCR conditions: 95°C × 10 min (94°C × 1 min, 60°C × 2

15   min, 72°C × 2 min) × 35 cycle, 72°C × 6 min).

        (2) A plasmid pRA-XX obtained by improving a

multicloning site of pLUCK (Biochem Biophys Res

Commun. 218, pp682, 1996) as shown in FIG. 11 was

prepared as a vector for protein expression.

20   (Subsequent to HindIII, a nucleotide sequence

encoding pelB as a signal peptide was arranged. Then,

restriction enzyme sites were arranged between NcoI

and EcoRI in order of NheI/SacII/SpeI. A nucleotide

sequence encoding His × 6 was arranged between

25   SacII/SpeI. In addition, ampicillin resistant gene,

T7 promoter, lac operator, and T7 terminator

sequences were the same as that of pluck).

(3) The PCR product and the plasmid were cleaved by using a restriction enzyme in NcoI/NheI (each available from New England Biolabs) in accordance with a recommended method.

5   The solution of the plasmid cleaved with the restriction enzyme was subjected to spin column 400HR (Amasham Science).

(4) The solution of the PCR fragments cleaved with the restriction enzyme was purified by using a

10   commercially available gel purification kit (SV Gel and PCR Clean-up system: Promega).

(5) The above 2 fragments were mixed with a commercially available T4 ligase kit (Roche) in accordance with a method recommended by a supplier,

15   followed by ligation. As a result, a VH coding gene insertion vector shown in Fig. 12A was obtained.

(6) Escherichia coli DS12S strain was transformed by using the ligation product through electropolation. A plasmid was prepared on a large

20   scale.

(7) Those plasmid solutions were serially diluted, and each solution was subjected to transformation of Escherichia coli BL21 (DE3) through electropolation. 700 μL of LB medium were added to

25   the solution, and the whole was cultured with shaking at 37°C for 1 hour. The culture solution was centrifuged at 6,000 rpm for 5 minutes, and then 650

μL of the supernatant were discarded.

(8) The remaining supernatant and a precipitate fraction were suspended, and the suspension was inoculated on an LB/Amp. plate. Then, the whole was left standing at 37°C overnight. As a result, an antibody VH library containing about 5×10^5 clones was eventually obtained.

(9) Next, 1,000 colonies were arbitrarily selected from the three 10^3-fold dilution plates, and a protein crude extract was prepared according to the following steps. Each colony was subcultured in 3 mL of a liquid medium of LB/amp., and the whole was cultured with shaking at 28°C for 6 hours. Next, IPTG was added to have a final concentration of 1 mM, and the whole was cultured with shaking for an additional 12 hours.

(10) Subsequently, a culture fraction and a supernatant bacterial fraction were obtained through centrifugation (10,500 rpm × 5 min).

(11) The resultant bacterial fraction was added with and suspended in 200 μL of an osmotic solution cooled in ice (0.5 M sucrose, 1 M Tris-HCl (pH 8.0), 0.5 mM EDTA), and the whole was left standing in ice for 10 minutes. Next, 1 mL of cooled sterilized water was added, and the whole was left standing in ice for 1 hour. After the resultant had been centrifuged (6,000 rpm × 30 min), the supernatant was

placed in a dialysis bag (MWCO 10,000) and was

dialyzed for 18 hours using Tris + 0.1% Tween 20

solution (20 mM Tris, 500 mM NaCl) as an external

solution while the external solution was changed

5   every 6 hours.

The dialysis internal solution obtained in the

above step was used as a sample for screening of a

gold-binding antibody heavy chain variable region

(VH).

10   Example 2 (Screening of gold-binding antibody

heavy chain variable region (VH))

A 96-well titer plate on which gold (having a

thickness of 100 nm) was deposited was prepared as a

substrate for screening of a gold-binding antibody

15   heavy chain variable region (VH). 250 µL of 1,000

sample solutions obtained in Example 1 were dispensed

in each well, and the plate was gently shaken for 1

hour. After the supernatant had been discarded, the

plate was turned upside down and was tapped on paper

20   towel 10 times to remove water. A washing step was

performed, which involved: adding 200 µL of Tris +

0.1% Tween 20 to each well; and gently shaking the

plate for 10 minutes. This operation was repeated 3

times. 200 µL of an antibody solution prepared by

25   diluting an HRP-binding anti-His antibody

(Invitrogen) with Tris + 0.1% Tween 20 solution at

1 : 10,000 were dispensed in each well, and the plate

was gently shaken for 1 hour. Subsequently, the same operation as the washing step was performed. 100 μL of each of an HRP substrate and Detect Reagents 1 and 2 (Amasham Science) serving as coloring materials were dispensed in each well, and the plate was gently shaken for 1 hour. The luminol chemiluminescence level was determined.

15 sample colonies in each of which chemiluminescence was observed as described above were subcultured in 1.5 mL of LB/amp., and the whole was cultured with shaking at 37°C overnight. A plasmid was purified from the resultant fungus by means of an SV MiniPrep DNA purification system (Promega). DNA sequences of 17 VL-representing phagemids obtained as described above were determined according to the following method. Primers for sequencing were set at a pelB sequence portion placed upstream of the VH coding gene of an expression vector. The primer for sequencing was as follows.

pelB-back

(SEQ ID No: 88) 5'-ccgct ggatt gttat tactc gc-3'

A BigDye-PCR reaction was performed by using the above primer and a sequencing reaction kit and a reaction solution composition recommended by a supplier. A temperature cycle was 96°C × 3 min → (94°C × 1 min → 50°C × 1 min → 68°C × 4 min) × 30 cycle. Next, the base sequence of the PCR product

purified through ethanol precipitation was determined

by means of a sequencer (377 manufactured by ABI).

As a result, each sequence of SEQ ID Nos.: 58 to 74

was obtained.

5   Example 3 (Preparation of antibody light chain

variable region VL library)

A VL gene library was prepared from a Fab

library of human peripheral blood cells in the same

manner as in Example 1. The primers were as follows.

10 ·back primer

(SEQ ID No: 89)

5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCGMCATYCAGWTGACCCAGTCT

CC-3'

(SEQ ID No: 90)

15 5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCGATRTTGTGATGACYCAGWCT

CC-3'

(SEQ ID No: 91)

5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCGAAATTGTGWTGACGCAGTCT

CC-3'

20 (SEQ ID No: 92)

5'-TCGCAACTGCGGCCCAGCCGGCCATGGCCGACATCGWGHTGACCCAGTCT

CC-3'

·forward primer

(SEQ ID No: 93)

25 5'-TTCTCGACTTGCGGCCGCACGTTTGATTTCCACCTT GGTCCC-3'

(SEQ ID No: 94)

5'-TTCTCGACTTGCGGCCGCACGTTTGATCTCCAGCTTGGTCCC-3'

(SEQ ID No: 95)

5'-TTCTCGACTTGCGGCCGCACGTTTGATATCCACTTT GGTCCC-3'

(SEQ ID No: 96)

5'-TTCTCGACTTGCGGCCGCACGTTTGATCTCCACCTT GGTCCC-3'

5 (SEQ ID No: 97)

5'-TTCTCGACTTGCGGCCGCACGTTTAATCTCCAGTCG TGTCCC-3'

In addition, a plasmid to be used was similarly
prepared except that NheI between NcoI/SacII was
changed to NotI at the time of preparation of a
10 plasmid for protein expression in (2) of Example 1.
Furthermore, the VL protein dialysis internal
solution obtained at the end of the step of (11) of
Example 1 was used as a sample for screening of a
gold-binding antibody light chain variable region
15 (VL).

Example 4 (Screening of gold-binding antibody
light chain variable region (VL))

Screening of a gold-binding VL was performed in
the same manner as in Example 2 except that the
20 sample obtained in Example 3 was used as a screening
sample. As a result, each sequence of SEQ ID Nos.:
75 to 77 was obtained.

Hereinafter, a phagemid was prepared from the
human peripheral blood cell Fab gene library of
25 Example 1, and a gold-binding VL or VH was screened.

Example 5 (Screening of gold-philic VL-
representing phage group)

A VL-representing phage library was prepared according to the following procedure and a phage group capable of binding to gold was selected.

(1) Phagemid for VL representing

5   By using a Fab library derived from human adult peripheral blood B lymphocyte as a template, VL coding gene was replicated by means of the primers of SEQ ID Nos. 89 to 97. Furthermore, part of the N terminal of PIII protein serving as a coat protein of

10   M13 phage was deleted, and a library of VL-representing phagemid (Fig. 12B) prepared in such a manner that VL protein was fused and expressed was used (Biochem Biophys Res Commun. 1996, 218, pp682).

(2) Preparation of VL fragment representing

15   phage library

1) Transformation

1 µL of the VL coding gene library (350 ng/µL) was transformed into 40 µL of Escherichia coli DH 12S through electropolation (applied voltage: 1.5 KV,

20   resistance: 186 Ω, capacity: 50 µF). Next, a VL-representing phage library was prepared according to the following procedure.

2) Culture

(i) 800 µL of LB medium were added to the DH12S

25   solution after the transformation, and the whole was cultured with shaking at 37°C for 1 hour (140 rpm).

(ii) 20 mL of the culture solution were added

to LB medium + ampicillin having a final
concentration of 100 µg/mL (LB/amp.), and the whole
was cultured at 37°C for 3 to 4 hours.

(iii) 40 µL of M13KO7 as a helper phage were
5   added, and the whole was cultured with shaking at 100
rpm for an additional one hour.

(iv) A 50 mg/mL kanamycin solution was added to
have a final concentration of 50 µg/mL, and the whole
was cultured with shaking at 37°C (100 rpm).

10   3) Collection of VL-representing phage library

(i) 5 mL of a 20% PEG/500 mM NaCl were added to
the culture solution, and the whole was left standing
in ice for one or more hours.

(ii) The supernatant was carefully removed
15   through centrifugation (6,500 rpm × 35 min).

(iii) The precipitate was suspended in 500 µL
of PBS buffer to prepare a VL-representing phage
solution.

4) Titer evaluation of VL-representing phage library

20   (i) 10 µL of JM109 glycerol stock were added to
LB, and the whole was cultured with shaking at 37°C.

(ii) The VL-representing phage solution
prepared in 3) was serially diluted with LB medium.

(iii) 10 µL of the serially diluted ($\times 10^{-6}$ to
25   10^{-10}) solution were added to 750 µL of (a) the
culture solution having an OD_{600} of about 0.5, and the
whole was cultured with shaking at 37°C for 1 hour.

(iv) Centrifugation (6,000 rpm × 5 min) was performed to remove 700 μL of the culture supernatant.

(v) The remaining culture supernatant and a precipitate were suspended by pipetting. Then, the

5   suspension was inoculated on an LB/amp. plate, and the whole was left standing at 37°C overnight.

(vi) The number of colonies emerged on a diluted concentration plate having 100 or less colonies was counted and defined as a titer value of

10   the VL-representing phage library.

The resultant VL-representing phage library solution: 5×10^9 cfu/μL

(3) VL panning using gold fine particles

The phage library solution prepared as

15   described above and gold fine particles (1.5 μmΦ: manufactured by Sigma-Aldrich) were used to repeat a panning operation according to the following method 5 rounds for the purpose of selecting a phage group representing a gold-binding VL.

20   1) Binding experiment

(i) 10 μL of a solution of the gold fine particles (50 mg/PBS 1 ml) and PBS + 0.1% Tween 20 (PBST) were added to the phage solution (in a volume such that the total number of phages in the solution

25   is 1,010 cfu) in a sterilized Eppendorf tube (1.5 mL) in such a manner that the total volume would be 1,000 μL, and the resultant was prepared as a binding

reaction solution.

(ii) The binding reaction solution was maintained for 30 minutes while being gently rotated at room temperature.

5   (iii) The resultant obtained in (ii) was centrifuged (10,000 rpm × 5 min), and the solution supernatant was carefully discarded.

2) Washing (washing of non-specific adsorbate)

(i) 500 μL of PBST were added to the gold fine
10 particles in the Eppendorf tube, and the whole was maintained for 10 minutes while being gently rotated at room temperature.

(ii) Centrifugation (10,000 rpm × 5 min) was performed, and the washing supernatant was carefully
15 removed.

(iii) Each of the above (i) and (ii) was repeated 10 times.

3) Acid elution and titer evaluation of acid eluted fraction

20   The phage titer adsorbed to the gold fine particles obtained in 2) was evaluated according to the following procedure.

3)-1 Acid elution

(i) 115 μL of 0.2 M Gly-HCl (pH 2.2) were added
25 to the gold fine particles after the washing, and the whole was maintained for 1 min while being rotated vertically.

(ii) Centrifugation (10,000 rpm × 5 min) was performed, and the supernatant was collected as an acid eluted fraction.

(iii) The collected acid eluted fraction was
5   neutralized by adding 15 µL of 1 M Tris-HCl.

(iv) 1 µL of the acid eluted solution after the neutralization was serially diluted, and a titer value was measured according to the titer evaluation method.

10   (v) The remaining acid eluted fraction was quickly mixed with a fraction of gold fine particles, and the mixture was suspended again.

3)-2 Titer evaluation

Titer evaluation was performed in the same
15   manner as in (2)-3), 4) to obtain the following results.

1st round: 9.8×10^2 cfu

2nd round: 1.0×10^3 cfu

3rd round: 7.8×10^2 cfu

20   4th round: 1.3×10^3 cfu

5th round: 1.1×10^4 cfu

4) Reinfection and phage amplification

Up to a fourth round, the phage group adsorbed to the gold fine particles obtained in 3) was
25   reinfected with Escherichia coli to amplify the number of phages for preparing a phage solution for subsequent panning.

(i) Escherichia coli JM109 was cultured with shaking at 37°C (140 rpm) in 20 mL of LB medium for reinfection and phage amplification.

(ii) The suspension of the gold fine particles

5 obtained in 3) was added to the Escherichia coli culture solution in (a) having an OD_{600} in the range of 0.3 to 0.5, and the whole was cultured with shaking at 37°C for 1 hour (140 rpm).

(iii) Ampicillin was added to have a final

10 concentration of 100 µg/mL, and the whole was cultured with shaking at 37°C for 2 hours.

(iv) 40 µL of helper phage M13KO7 were added, and the whole was cultured at 37°C for 1 hour while a shaking speed was reduced to 100 rpm.

15 (v) Kanamycin was added to have a final concentration of 50 µg/mL, and the whole was cultured with shaking at 37°C overnight.

(vi) A phage in the culture supernatant was collected by the same operation as those of (2)-3),

20 4) to prepare a phage solution. Furthermore, the phage solution was evaluated for titer values to confirm that the phage was amplified.

The titer values obtained after amplification are shown below.

25 1st round: 2.4×10^{9} cfu

2nd round: 8.1×10^{8} cfu

3rd round: 1.8×10^{9} cfu

4th round: 1.1×10^{10} cfu

(4) Phage ELISA

1) Preparation of gold-deposited substrate for ELISA

The same 96-well titer plate (BD, polystyrene)

5   on which gold (having a thickness of 100 nm) was

deposited as that of Example 2 was used as a

substrate for a phage ELISA.

2) Preparation of VL-representing phage monoclone

A phagemid was collected from 11 colonies

10   expressing on a 10^4-fold diluted plate in the titer

evaluation on a fifth round in (3)-4) according to

the following procedure.

(i) Each colony was cultured at 37°C with 20 mL

of LB/amp.

15   (ii) 40 μL of helper phage M13KO7 were added,

and the whole was cultured at 37°C for 1 hour while a

shaking speed was reduced to 100 rpm.

(iii) Kanamycin was added to have a final

concentration of 50 μg/mL, and the whole was cultured

20   with shaking at 37°C overnight.

(iv) A phage in the culture supernatant was

collected by the same operation as those of (2)-3),

4) to prepare a phage solution. Furthermore, the

phage solution was evaluated for titer value to

25   confirm that the phage was amplified.

Titer values obtained after amplification are

shown below.

No. 1:3.8×10^{9}cfu

No. 2:9.0×10^{8}cfu

No. 3:2.4×10^{9}cfu

No. 4:1.0×10^{9}cfu

5   No. 5:9.7×10^{7}cfu

No. 6:4.4×10^{8}cfu

No. 7:6.2×10^{9}cfu

No. 8:8.9×10^{7}cfu

No. 9:1.4×10^{9}cfu

10   No. 10:1.1×10^{10}cfu

No. 11:4.9×10^{9}cfu

3) Preparation of phage solution

Each phage solution obtained in 2) was used to

prepare 200 μL of each of dilution series in which

15   the titer value was sequentially reduced from 10^9 cfu

by a factor of 10 according to the following

composition.

VL-representing phage solution: x μL

Super blocking buffer (PIERCE): 20 μL

20   0.5% Tween 20/PBS: x/5 μL

PBS: 180 - (6x/5) μL

4) ELISA

(i) 80 μL of each of the serially-diluted VL-

representing phage solutions were dispensed in a

25   gold-deposited titer plate, and the plate was gently

shaken for 1 hour in a shaker.

(ii) The phage solution was removed, 90 μL of

PBST were dispensed in each well, and the whole was
stirred for 10 minutes, followed by removing the
washing supernatant. This operation was repeated 3
times.

5   (iii) 75 µl of a solution of HRP-Anti M13
immunoglobulin/SBB/PBS (1/1,000 : 1 : 10) were
dispensed in each well, and the plate was gently
shaken for 1 hour in a shaker.

          (iv) The supernatant of the immunoglobulin
10   solution was discarded. Next, PBST was dispensed in
a volume of 90 µL/well, and the whole was stirred for
10 min. This washing operation was repeated 3 times.

          (v) Each of detection reagents 1 and 2 (Amasham
BIOSCIENCE) was dispensed in a volume of 35 µL/well,
15   and the whole was allowed to react while being gently
stirred for 1 min.

          (vi) The luminescence intensity of luminol was
measured. No. 1, No. 2, and No. 3 having high
luminescence intensities were provided as gold-
20   binding VL-representing phage clones.

          Phagemids were isolated from the above four
phage clones to elucidate the base and amino acid
sequences of a gold-binding VL. Next, an expression
vector was prepared to express a protein, thereby
25   confirming binding affinity on a surface plasmon
resonance (SPR) gold substrate.

          Example 6 (Acquisition of gold-philic VL

protein)

(1) Collection of phagemid

A phagemid was collected from the colony
corresponding to No. 7 expressing on a 10^4-fold

5   diluted plate in the titer evaluation on the fifth
round in (3)-4) of Example 5 according to the
following procedure.

(i) Each colony was cultured at 37°C overnight
with 1.6 mL of LB/amp.

10   (ii) A phagemid was collected in accordance
with a method recommended by a supplier by using a
MiniPrep SV plus DNA Purification system (promega).

(2) Preparation of expression vector

Expression vectors expressing the above 3 kinds

15   of VL proteins were constructed according to the
following configurations. pRA-XX shown in FIG. 11 of
Example 1 and the phagemid obtained in (1) were
cleaved through a restriction enzyme reaction by
using NcoI and NotI of each of them. The resultant

20   VL fragment was inserted into pRA-XX to prepare a
plasmid pRA-VLNo, n (n: clone number) expressing VL
code nucleic acid as a fusion protein (FIG. 13).

(3) Expression and purification of protein

3 vectors for expressing VL proteins obtained

25   as described above were used to express VL proteins.

1) Transformation

The above expression vectors were used to

transform 40 µL of BL21 (DE3) competent cell. The

transformation was performed under such a condition

that heat shock was performed in ice → 42°C × 90 sec

→ in ice. 750 µL of LB medium were added to the BL21

5 solution transformed by heat shock, and the whole was

cultured with shaking for 1 hour at 37°C. After that,

centrifugation was performed at 6,000 rpm × 5 min,

and 650 µL of the culture supernatant were discarded.

The remaining culture supernatant and a cell fraction

10 as a precipitate were stirred and inoculated on an

LB/amp. plate, and the whole was left standing at

37°C overnight.

2) Preculture

A colony on the plate was selected at random

15 and was cultured with shaking at 28°C overnight in

3.0 mL of an LB/amp. medium.

3) Main culture

The preculture solution was subcultured in 750

ML of a 2 × YT medium, and the culture was continued

20 at 28°C. When OD_{600} exceeded 0.8, IPTG was added to

have a final concentration of 1 mM, and culture was

further performed at 28°C overnight.

4) Purification

(i) Ammonium sulfate precipitation

25 The culture solution obtained in 3) was

centrifuged at 6,000 rpm × 30 min to obtain a culture

supernatant. The weight of the resultant culture

supernatant was measured. Then, ammonium sulfate in
a weight of 60% of the weight of the culture
supernatant was gradually added. Then, the resultant
was stirred at 4°C overnight.

5   (ii) Desalting

The solution obtained in (i) was centrifuged at
8,000 rpm × 20 min, and the supernatant was discarded.
The resultant precipitate was added with and immersed
in 15 mL of 20 mM Tris/500 mM NaCl (hereinafter,

10 referred to as a Tris solution) at 4°C overnight for
dissolution. Next, the resultant solution was
charged into a cellulose tube for dialysis
(manufactured by Sanko Junyaku Co., Ltd.) and was
dialyzed at 4°C by using the Tris solution as an

15 external solution for desalting (the external
solution was changed every 6 hours).

(iii) Metal chelate column

A His-Bind (manufactured by Novagen) was used
as a metal chelate column carrier. Column adjustment,

20 sample loading, and a washing step were performed at
4°C in accordance with the method recommended by the
supplier. Elution of a His tag-fused VL protein as a
target was performed with a 500 mM imidazole/Tris
solution.

25 SDS-PAGE (acrylamide 15%) of the eluent
confirmed that the eluent had a single band and was
purified. The eluent was dialyzed again by using the

'Tris solution as an external solution to remove

imidazole in the eluent. Furthermore, buffer

substitution was performed by changing the external

solution to a phosphate buffer (hereinafter, referred

5   to as PBS) to prepare a VL protein solution for SPR.

Example 7 (SPR measurement)

The binding affinity of the VL protein obtained

in Example 6 for gold was measured by means of SPR.

A BIAcore 2000 (manufactured by BIAcore) was used as

10   an SPR measuring device. A gold-deposited glass

substrate SIA-kit Au of the same company was used as

a gold substrate to which the protein was to be bound.

Measurement was performed under the following

conditions.

15   Running buffer: PBST

Temperature: 25°C

Flow rate: 20 µL/min

Sample: VL protein/PBST

Injection amount: 20 µL

20   A binding curve in which the binding affinity

was confirmed was obtained (Fig. 9).

Example 8 (Determination of base and amino acid

sequences of VL protein)

The DNA sequence of the VL No, 7 obtained as

25   described above was determined in accordance with the

following method.

A primer for sequencing was set at a pelB

sequence portion placed upstream of the VL coding

gene. The primer for sequencing was as follows.

pelB-back

(SEQ ID No: 88)

5   5'-ccgct ggatt gttat tactc gc-3'

A BigDye-PCR reaction was performed by using

the above primer in the same manner as in Example 2

and a sequencing reaction kit and a reaction solution

composition recommended by a supplier. A temperature

10   cycle was 96°C × 3 min → (94°C × 1 min → 50°C × 1 min

→ 68°C × 4 min) × 30 cycle → 4°C. Next, the base

sequence of the PCR product purified through ethanol

precipitation was determined by means of a sequencer

(377 manufactured by ABI). The following result was

15   obtained. No. 7 had the same base sequence as that

of SEQ ID No: 76 of Example 12.

Example 9 (Screening of gold-philic VH-

representing phage group)

A VH-representing phage library was prepared

20   according to the following procedure to select a

phage group having a binding affinity for gold.

(1) Phagemid for representing VH

A Fab library derived from human adult

peripheral blood B lymphocyte was used as a template

25   in the same manner as in Example 1, and the primers

of SEQ ID Nos. 80 to 89 were used in the same manner

as in Example 1. Thus, VH coding gene was replicated.

Furthermore, part of the N terminal of PIII protein serving as a coat protein of M13 phage was deleted, and a library of VH-representing phagemid (FIG. 12A) prepared in such a manner that VH protein was fused

5   and expressed was used.

(2) Preparation of VH-representing phage library

A VH-representing phage library was prepared in the same manner as in (2) of Example 5 except that the product obtained in (1) was used. The resultant

10 VH-representing phage library solution: 1×10^9 cfu/μL.

(3) VH panning using gold fine particles

Panning for selecting a phage group representing a gold-binding VH was performed in the

15 same manner as in (3) of Example 5 except that the phage library solution prepared in (2) above was used.

1) Binding experiment

Hereinafter, 2) Washing and 3) Acid elution and titer evaluation similar to those of Example 5 were

20 performed.

1st round: 9.8×10^2 cfu

2nd round: 1.0×10^3 cfu

3rd round: 7.8×10^2 cfu

4) Reinfection and phage amplification

25 Reinfection and phage amplification were performed in the same manner as in Example 5. Titer values obtained after amplification are shown below.

1st round: 2.4×10^9cfu

2nd round: 8.1×10^8cfu

(4) Phage ELISA

1) Preparation of gold-deposited substrate for ELISA

5   The same substrate as that of Example 5 was used as a substrate for phage ELISA.

2) Preparation of VL-representing phage monoclone

    A phagemid was collected from 20 colonies expressing on a 10^4-fold diluted plate in the titer

10 evaluation on a third round in the VH panning operation in the same manner as in Example 1. The titer values of the resultant VH-representing phage monoclone solutions are shown below.

No. 1 : 3.8×10^9cfu

15 No. 2 : 9.0×10^8cfu

No. 3 : 2.4×10^9cfu

No. 4 : 1.0×10^9cfu

No. 5 : 9.7×10^7cfu

No. 6 : 4.4×10^8cfu

20 No. 7 : 6.2×10^9cfu

No. 8 : 8.9×10^7cfu

No. 9 : 1.4×10^9cfu

No. 10 : 1.1×10^{10}cfu

No. 11 : 4.9×10^9cfu

25 No. 12 : 9.0×10^8cfu

No. 13 : 2.4×10^9cfu

No. 14 : 1.0×10^9cfu

No. $15 : 9.7 \times 10^{7}$ cfu

No. $16 : 4.4 \times 10^{8}$ cfu

No. $17 : 6.2 \times 10^{9}$ cfu

No. $18 : 8.9 \times 10^{7}$ cfu

5   No. $19 : 1.4 \times 10^{9}$ cfu

No. $20 : 1.1 \times 10^{10}$ cfu

3) Preparation of phage solution

        Each phage solution obtained in 2) was used to
prepare 200 µL of each of dilution series in which

10   the titer value was sequentially reduced from 10^{9} cfu
by a factor of 10 according to the following
composition.

VL-representing phage solution: x µL

Soluble VL solution (50 ng): x µL

15   Super blocking buffer (PIERCE): 20 µL

0.5% Tween 20/PBS: x/5 µL

PBS: 179 - (6x/5) µL

4) Phage ELISA

        Phage ELISA was performed by the same operation

20   as that of Example 1. Three clones each having a
luminescence intensity higher than that of a VH
library solution for comparison were provided as
gold-binding VH-representing phage clones (Nos. 2, 4,
and 6).

25   Example 10 (Acquisition of gold-philic VH
protein)

        Phagemids were isolated from the above three

phage clones, and then their expression vectors were
prepared to express a protein, thereby confirming
binding affinity on a surface plasmon resonance (SPR)
gold substrate. Furthermore, the base and amino acid
5   sequences of each of those gold-binding VH were
elucidated.

(1) Collection of phagemid

A phagemid was collected from the colony
corresponding to 15 clones expressing on a 10^4-fold
10   diluted plate in the titer evaluation on the third
round according to the following procedure.

(a) Each colony was cultured at 37°C overnight
in 1.6 mL of LB/amp..

(b) A phagemid was collected in accordance with
15   a method recommended by a supplier by using a
MiniPreps SV plus DNA Purification system (promega).

(2) Preparation of expression vector

Expression vectors expressing the above 3 kinds
of VL proteins were constructed according to the
20   following configurations.

pUT-XX was prepared by changing a multicloning
site of PET-15b (Novagen). The pUT-XX and the
phagemid obtained in (1) above were cleaved through a
restriction enzyme reaction by using NcoI and NotI of
25   each of them. The resultant VH fragment was inserted
into pUT-XX to prepare a plasmid pRA-7sn (n: phage
clone number described above) expressing VH coding

nucleic acid as a fusion protein (Fig. 14).

(3) Expression and purification of protein

3 kinds of VHs (7s2, 7s4, and 7s6) were
acquired by performing the above procedure (treating
5   the expression vectors in the step of protein
expression and purification described below in an
individual system).

1) Transformation

The above 2 expression vectors were used to
10   transform 40 µL of different BL21 (DE3) competent
cell solutions. The transformation was performed
under such a condition that heat shock was performed
in ice → 42°C × 90 sec → in ice. 750 µL of LB medium
were added to the BL21 solution transformed by heat
15   shock, and the whole was cultured with shaking for 1
hour at 37°C. After that, centrifugation was
performed at 6,000 rpm × 5 min, and 650 µL of the
culture supernatant were discarded. The remaining
culture supernatant and a cell fraction as a
20   precipitate were stirred and inoculated on an LB/amp.
plate, and the whole was left standing at 37°C
overnight.

2) Preculture

A colony on the plate was selected at random
25   and was cultured with shaking at 28°C overnight with
3.0 mL of an LB/amp. medium.

3) Main culture

The preculture solution was subcultured in 750
ML of a 2 × YT medium, and the culture was further
continued at 28°C. When OD_{600} exceeded 0.8, IPTG was
added to have a final concentration of 1 mM, and
5   culture was performed at 28 °C overnight.

4) Purification

A target polypeptide chain was purified from an
insoluble granule fraction through the following
steps.

10   (i) Collection of insoluble granule

The culture solution obtained in 3) above was
centrifuged at 6,000 rpm × 30 min to obtain a
precipitate as a bacterial fraction. The resultant
was suspended in a Tris solution (20 mM Tris/500 mM
15   NaCl) in ice. The resultant suspension was
homogenized with a French press to obtain a
homogenized solution. Next, the homogenized solution
was centrifuged at 12,000 rpm × 15 min, and the
supernatant was removed to obtain a precipitate as an
20   insoluble granule fraction.

(ii) Solubilization of insoluble granule fraction

The insoluble fraction obtained in (i) was
added with and immersed overnight in 10 mL of a 6 M
guanidine hydrochloride/Tris solution. Next, the
25   resultant was centrifuged at 12,000 rpm × 10 min to
obtain a supernatant as a solubilized solution.

(iii) Metal chelate column

A His-Bind (manufactured by Novagen) was used as a metal chelate column carrier. Column adjustment, sample loading, and a washing step were performed at room temperature (20°C) in accordance with the method

5   recommended by the supplier. Elution of a His tag-fused VL polypeptide as a target was performed in a 60 mM imidazole/Tris solution. SDS-PAGE (acrylamide 15%) of the eluent confirmed that the eluent had a single band and was purified.

10 (iv) Dialysis

The eluent was dialyzed by using a 6 M guanidine hydrochloride/Tris solution as an external solution at 4°C to remove imidazole in the eluent, thereby obtaining the above polypeptide chain

15 solutions.

(v) Refolding

In the same manner as that described above, the polypeptide chain solutions of VHg-VLh and VHh-VLg were separately dialyzed according to the following

20 steps for removing guanidine hydrochloride (4°C) to perform refolding of a protein.

a) A sample having a concentration of 7.5 µM (volume after dilution of 10 ml) was prepared by using a 6 M guanidine hydrochloride/Tris solution on the basis of

25 the molar absorbance coefficient and ΔO.D. (280 nm-320 nm) level of each polypeptide chain. Next, β-mercaptoethanol (reductant) was added to have a final

concentration of 375 µM (50-fold protein

concentration) for reduction at room temperature in a

dark room for 4 hours. The sample solution was

charged into a dialysis bag (MWCO: 14,000) and

5   provided as a sample for dialysis.

b) The sample for dialysis was immersed in a 6 M

guanidine hydrochloride/Tris solution as an external

solution, and was dialyzed for 6 hours while being

gently stirred.

10   c) The concentration of the guanidine hydrochloride

solution of the external solution was reduced to 3 M

and then to 2 M in a stepwise manner. The sample was

dialyzed at each concentration of the external

solution for 6 hours.

15   d) The Tris solution was added with oxidized

glutathione (GSSG) to have a final concentration of

375 µM and with L-Arg to have a final concentration

of 0.4 M), and then was added with the 2 M dialysis

external solution in 3) above to have a guanidine

20   hydrochloride concentration of 1 M. The sample was

dialyzed in the external solution whose pH had been

adjusted to 8.0 (4°C) with NaOH for 12 hours while

being gently stirred.

e) An L-Arg Tris solution having a guanidine

25   hydrochloride concentration of 0.5 M was prepared by

the same operation as that of d), and dialysis was

performed for an additional 12 hours.

f) Finally, dialysis was performed for 12 hours in a
Tris solution. After the completion of the dialysis
7), centrifugation was performed at 10,000 rpm for
about 20 min to separate an aggregate and a

5   supernatant.

The 3 kinds of VH solutions obtained as
described above were subjected to buffer substitution
by changing the external solution to a phosphate
buffer (hereinafter, referred to as PBS) to prepare

10   VH protein solution for SPR.

Example 11 (SPR measurement)
The binding affinity of the VH protein obtained
in Example 10 for gold was measured by means of SPR
in the same manner as in Example 7. A binding curve

15   in which the binding affinity was confirmed was
obtained for each of 7s2, 7s4, and 7s6. FIG. 10
shows the representative examples). The following
K_D's were obtained by curve fitting.
7s2: $K_D = 5.0 \times 10 \ M^{-8}$

20   7s4: $K_D = 8.0 \times 10 \ M^{-9}$
7s6: $K_D = 3.0 \times 10 \ M^{-7}$

Example 12 (Determination of base and amino
acid sequences of VH protein)
The DNA sequence of each of the three VH-

25   representing phagemids obtained as described above
was determined in the same manner as in Example 2. A
primer for sequencing was set at a pelB sequence

portion placed upstream of the VH coding gene. The
primer for sequencing a s that of Example 2 was used,
and analysis was performed according to the same
procedure to obtain three different sequences. Those
5   sequences obtained in Example 10 corresponded to SEQ
ID Nos.: 59 to 61.

     Example 13 (Gold-binding experiment on VH/VL
composite by means of SPR)

     A PBST solution containing 50 nM of each of the
10   VL clone No. 7 obtained in Example 6 and the VH
clone: 7s2 obtained in Example 10 was prepared and
preserved at 4°C for one day. SPR measurement was
performed by using the mixed solution in the same
manner as in Example 7. As a result, binding
15   affinity for gold was confirmed at a lower
concentration than that of Example 7 or Example 11.
This result suggests that mixing of the clones forms
a complex (Fv) to increase binding affinity owing to
its stabilized structure (FIG. 3).

20   Example 14 (Acquisition of gold-binding scFv)
     An scFv composed of the VL clone: No. 7
obtained in Example 6 and the VH clone: 7s2 obtained
in Example 10 was prepared according to the following
procedure.

25   (1) Preparation of expression vector
     An expression vector which could express a
fusion protein, which could be continuously

translated from VL (No. 7) coding gene, linker

(GGGGS) × 3, VH (7s4) coding gene, and His × 6

(hereinafter, referred to as His tag).

A specific preparation method is shown in FIG.

5   15.

The following primers were used.

scFv-B (SEQ ID No: 117)

5'-NNNNNCCATGGCCGGGGGCGGGGGCAGCGGGGGCGGGGGCAGCGGGGG

CGGGGGCAGCCAGGTGCAGTTGGTGGAGTCT-3'

10   scFv-F (SEQ ID No: 118)

5'-NNNNNCCGCGGAACCATTCAGATCCTCTTCT-3'

(2) Expression and purification of protein

Hereinafter, the vector for expressing an scFv

protein obtained as described above was used to

15   express and purify an scFv protein.

1) Protein expression

The above expression vector was used to

transform Escherichia coli BL21 (DE3), and culture

was performed by using a 2xYT medium at 28°C.

20   Expression was induced with IPTG having a final

concentration of 1 mM at O.D.$_{600}$ = about 0.8, and the

resultant was cultured with shaking overnight. The

bacterial body was centrifuged at 6,000 rpm × 20 min

to obtain a culture supernatant fraction and a

25   bacterial fraction. Those samples were

electrophoresed according to a conventionally known

method by means of SDS-PAGE to confirm the expression

amount of a target protein. The result showed that
secretion into the culture supernatant fraction was
extremely small. In view of the above, in order to
purify the target protein from the bacterial fraction,

5   a sample was prepared according to the following
procedure. First, the bacterial body was resuspended
in 15 mL of PBS, the suspension was further added
with 25 mL of PBS, and the mixture was homogenized by
means of a French press. The resultant homogenized

10   solution was centrifuged at 12,000 rpm × 15 min to
obtain a precipitate fraction as insoluble granules.
The resultant insoluble granules were immersed in a 6
M guanidine hydrochloride/Tris solution overnight and
solubilized to obtain a sample for metal chelate

15   column.

2) Purification by metal chelate column

        A 6 M guanidine hydrochloride/5 mM
imidazole/Tris solution was used as a running buffer,
and purification was performed in the same manner as

20   in Example 2 except that: the imidazole concentration
at the time of elution was set to 100 mM; and a
developing temperature was set at room temperature
(20°C).

3) Dialysis

25   The eluted fraction obtained in 2) was charged
into a cellulose tube for dialysis (manufactured by
Sanko Junyaku Co., Ltd.) and was dialyzed at 4°C by

using a 6 M guanidine hydrochloride/1 mM EDTA/Tris

solution as an external solution (the external

solution was changed every 6 hours).

4) Reconstruction of protein

5   An scFv solution obtained in 3) was adjusted

with Tris solution to 7.5 µM to be provided as a

sample. An scFv structure was reconstructed while

the guanidine hydrochloride concentration gradually

in the internal solution was reduced by reducing the

10   glycin hydrochloride concentration in the external

solution with respect to the above solution.

(a) A sample having a concentration of 7.5 µM of a

molar absorbance coefficient and of an O.D. (280 nm)

estimated from the amino acid sequence of the target

15   protein was prepared by using a 6 M guanidine

hydrochloride/Tris solution.

(b) Next, β-mercaptoethanol (reductant) was added to

have a final concentration of 375 µM, followed by

reduction at room temperature in a dark room for 4

20   hours.

(c) The sample was charged into the same dialysis bag

as that described above, and the whole was placed in

an external solution (a 6 M guanidine

hydrochloride/Tris solution) and dialyzed at 4°C for

25   about 6 hours.

(d) The external solution was changed twice

thereafter every 6 hours. At the time of such change,

the sample was dialyzed while the concentration of
the guanidine hydrochloride solution of the external
solution was reduced to 3 M and then to 2 M in a
stepwise manner.

5   (e) Next, the Tris solution was added with oxidized
glutathione (GSSG) to have a final concentration of
375 µM and with L-Arg to have a final concentration
of 0.4 M), and then was added with the dialysis
external solution (2 M) in (d) above to have a

10 guanidine hydrochloride concentration of 1 M. The
sample was dialyzed in an external solution whose pH
had been adjusted to 8.0 (at 4°C) with NaOH for about
12 hours.

(f) A 0.5 M guanidine hydrochloride/Tris solution was

15 prepared by the same operation as that of (e), and
dialysis was performed at 4°C for about 12 hours.
(g) Finally, dialysis was performed 4°C for about 12
hours while the external solution was changed to in a
Tris solution.

20 (h) After the completion of the dialysis, the
resultant was centrifuged at 10,000 rpm for about 20
min to separate an aggregation fraction and a
supernatant fraction. The supernatant fraction was
subjected to SDS-PAGE electrophoresis to confirm that

25 the target protein was made soluble in the
supernatant fraction. As a result, an scFv having
the No. 7 clone in VL and 7s2 in VH was acquired.

Example 15 (Evaluation of scFv for binding
affinity for gold by means of SPR)

The binding affinity of the scFv protein
obtained in Example 14 for gold was measured by means
5   of SPR in the same manner as in Example 8. A binding
curve showing binding affinity for gold was obtained
(FIG. 17).

Example 16 (Confirmation of Au specificity)

Gold specificity of the scFv protein obtained
10   in Example 15 was confirmed. Gold was deposited in a
circular pattern having a size of 70 $\mu m \varphi$ (having a
thickness of 50 nm) on a silicon substrate measuring
3 mm × 5 mm as a substrate for confirmation. The
surface of the substrate was sequentially immersed in
15   isopropyl alcohol, acetone, and hydrochloric acid for
10 min for each of the solutions for surface washing
(the surface was washed with deionized water and
dried every time the solution was changed). The
substrate was immersed in a solution prepared by
20   adjusting the scFv solution obtained in Example 6 to
1 μM for 1 hour. Subsequently, the substrate was
washed while being gently stirred for 10 min in PBST.
This operation was repeated 3 times and then a
washing solution was discarded. Next, the substrate
25   was immersed in a 100 nM anti-His tag antibody/PBST
solution while being gently stirred in the solution
for 1 hour. Subsequently, the substrate was washed

while being gently stirred for 10 min in PBST. This

operation was repeated 3 times and then a washing

solution was discarded. Furthermore, the substrate

was immersed in a 100 μM rhodopsin-binding anti-IGg

5   antibody/PBST solution while being gently stirred in

the solution for 1 hour. Subsequently, the substrate

was washed while being gently stirred for 10 min in

PBST. This operation was repeated 3 times and then a

washing solution was discarded. After that, the

10   substrate was observed with a fluorescence microscope.

As a result, fluorescence was observed in a circular

portion on which gold was deposited, and no

fluorescence was observed in a silicon portion.

Therefore, gold specificity of scFv obtained in

15   Example 15 was confirmed.

Comparative Example 1

The gold-deposited silicon substrate was

treated by the same operation as that of Example 16

except that the substrate was treated with 1 μM scFv.

20   The substrate was observed with a fluorescence

microscope, with the result that no fluorescence was

observed in each of a silicon portion and a circular

portion on which gold was deposited.

Example 17 (Preparation of silicon oxide-philic

25   peptide-fused gold-binding protein)

A protein having a silicon oxide-philic peptide

IPHVHHKHPHV on the C terminal of the scFv of the

above example was prepared according to the following

steps.

(1) Preparation of expression vector

(a) The pUT-scFv (VL#No, 7 × 7s4) obtained in the

5   above example (Example 14) was templated to perform

PCR by using the following primers.

SiscFv-B (SEQ ID No: 119)

5'-NNNNNCCATGGCCCAGGTGCAGTTGGTGGAGT-3'

SiscFv-F (SEQ ID No: 120)

10   5'-NNNNNCCGCGGCACGTGGGGGTGCTTGTGGTGCACGTGCATGGGGATA

ACCATTCAGATCCTCTTCT-3'

PCR was performed by using a commercially

available PCR kit (TAKARA BIO INC, LA-Tag kit) in

accordance with a protocol recommended by a supplier.

15   (b) The resultant PCR product was subjected to 2%

agarose electrophoresis. Next, about 400 bp of PCR

fragments were obtained from the gel through rough

purification by using a gel extraction kit (Promega).

The sequence confirmed that the fragments had a

20   target base sequence.

(c) pUT-scFv (7s4) and the PCR fragments obtained in

(b) above were cleaved by using NotI/SacII. Next,

agarose electrophoresis was performed to purify the

target fragments on each of a Vector side and an

25   Insert side.

(d) The purified nucleic acid fragments obtained in

(c) above were mixed at a ratio of Vector : Insert =

1 : 5, and then a ligation reaction was performed in

the same manner as in Example 1.

Hereinafter, transformation, collection of

plasmids, and confirmation of inserts were performed

5   in the same manner as in Example 6.

(3) Expression and purification of protein

The expression and purification of a protein

were performed in the same manner as in Example 10 by

using the plasmids for expression obtained as

10 described above, to thereby obtain a target protein.

Example 18 (Acquisition of gold- and HEL-

binding protein)

(1) Adjustment of gold-binding VH code nucleic acid

fragment

15   The following primers

gVH-B (SEQ ID No: 121)

5'-NNNNN CCATGG CCGAC CAGG TGCAG TTGGT GGAGT CT-3'

gVH-F (SEQ ID No: 122)

5'NNNNN GCTAG C GGAGA CGG TGACCAGGGT-3'

20 were used in order to prepare a gold-binding VH

(hereinafter, referred to as VHg) for introducing a

vector having a restriction enzyme NcoI cleavage site

arranged on a 5' terminal side of a gold-binding VH

(SEQ ID No: 61) and a restriction enzyme NheI

25 arranged on a 3' terminal side thereof. Then, PCR

was performed by using a commercially available PCR

kit at a proportion recommended by a supplier to

obtain about 350 bp of base pairs. The above VHB-F
was used to perform a BigDye-PCR reaction by using a
commercially available sequencing reaction kit and a
reaction solution composition. A temperature cycle

5   was set to 96°C × 3 min → (94°C × 1 min → 50°C × 1
min → 68°C × 4 min) × 30 cycle → 4°C to confirm that
fragments each having a base sequence encoding a
target VH were obtained.

(2) Adjustment of gold-binding VL code nucleic acid

10 fragments

    Nucleic acid fragments were obtained in the
same manner as in (1) except that the following
primers
gVL-B (SEQ ID No: 123)

15 NNNNN GCTAGC GGTGGCGGTGGCTCT GAAATTGTGTT GACGCAGTCT,
and
gVL-F (SEQ ID No: 124)
NNNNN CCGCG GCACG TTTAA TCTCC AGTCG TGT
were used in order to prepare a gold-binding VL

20 (hereinafter, referred to as VLg) (SEQ ID No: 99) for
inserting a vector having the restriction enzyme NheI
cleavage site and a nucleic acid encoding a linker
(GGGGS) arranged on a 5' terminal side of a gold-
binding VL (SEQ ID No: 76) and His × 6 and a

25 restriction enzyme SacII cleavage site arranged on a
3' terminal side thereof, to thereby confirm that the
fragments each having a base sequence of a target VL

were obtained.

(3) Adjustment of HEL-binding VH code nucleic acid
fragments

Nucleic acid fragments were obtained in the
5   same manner as in (1) except that the following
primers
·   hVH-B (SEQ ID No: 126)
5'-NNNNN CCATGG CCGAC GATATCCAGCTGCAGGAGTCGGGCCC-3',
and

10   hVH-F (SEQ ID No: 127)
5'NNNNN GCTAG C GGAGA CGG TGACGTCTGT-3
were used in order to prepare a HEL-binding VH
(hereinafter, referred to as VHh) for introducing a
vector having a restriction enzyme NcoI cleavage site
15   arranged on a 5' terminal side of a HEL-binding VH
(SEQ ID No: 125) and a restriction enzyme NheI
arranged on a 3' terminal side thereof, to thereby
confirm that the fragments had a base sequence of the
target VL.

20   (4) Adjustment of HEL-binding VL code nucleic acid
fragments

Nucleic acid fragments were obtained in the
same manner as in (1) except that the following
primers
25   hVL-B (SEQ ID No: 129)
NNNNNGCTAGCGGTGGCGGTGGCTCTGATATCGTCCTGACCCAGAG, and
hVL-F (SEQ ID No: 130)

`NNNNN CCGCG GCCTT GATCT CCAGC TTGGT GC`

were used in order to prepare an HEL-binding VL

(hereinafter, referred to as VLh) for introducing a

vector having the restriction enzyme NheI cleavage

5   site and a nucleic acid encoding a linker (GGGGS)

arranged on a 5' terminal side of an HEL-binding VL

(SEQ ID No: 128) and His × 6 and the restriction

enzyme SacII cleavage site arranged on a 3' terminal

side thereof, to thereby confirm that the fragments

10   had a base sequence of the target VL.

Example 19 (Preparation of expression vector)

The above 4 kinds of nucleic acid fragments

were used to constitute 2 expression vectors

according to the following configurations.

15   (1) Preparation of vector for expressing VHg-VLh

(pGHEL) (Fig. 15)

(i) Insertion of VHg

The plasmid pUT-XX was cleaved with restriction

enzyme NcoI/NheI (each manufactured by New England

20   Biolabs) and subjected to spin column 400HR (Amasham

Science). Next, VHg was similarly cleaved with the

restriction enzyme NcoI/NheI, and the cleaved

fragments were purified by using a commercially

available gel purification kit (SV Gel and PCR Clean-

25   up system: Promega). The above 2 fragments were

mixed with a commercially available T4 ligase kit

(Roche) in accordance with a method recommended by a

supplier, followed by ligation.

The ligation solution was transformed into 40
µL of JM109 competent cell (Promega) by means of heat
shock. The resultant was inoculated on an
5   LB/ampicillin (amp.) plate, and the whole was left
standing at 37°C overnight.

Next, an arbitrary colony on the plate was
subcultured in 3 mL of a liquid medium, and the whole
was cultured with shaking at 37°C overnight. After
10 that, plasmids were collected by using a commercially
available MiniPreps kit (Plus Minipreps DNA
Purification System: Promega).

The base sequence of the resultant plasmid was
confirmed by means of the sequence method by using
15 gVH-F. Thus, it was confirmed that the target
fragments were inserted.

(ii) Insertion of VLH

The plasmid pUT-VHg obtained in 1) above was
cleaved with a restriction enzyme NheI/SacII and
20 subjected to spin column 400HR (Amasham Science).
Next, VLh was similarly cleaved with the restriction
enzyme NheI/SacII. Next, ligation was performed in
the same manner as in (a) above to confirm that a
plasmids for expressing VHg-VLH were obtained (the
25 primer for confirmation was hVL-F).

(2) Preparation of vector for expressing VHh-VLg
(pHGOLD) (Fig. 16)

(iii) Insertion of VHh

VHh was inserted into a plasmid pUT in the same manner as in (i) above to confirm that the resultant plasmids were the target plasmids (the primer for

5 confirmation was hVH-F).

(iv) Insertion of VLg

VLg was inserted into the plasmids obtained in (iii) above in the same manner as in (b) above to confirm that the resultant plasmids were the target

10 vector pHGOLD for expressing VHH-VLg in the same manner as in 1) (the primer for confirmation was gVL-F).

Example 20 (Expression and purification of protein)

15 Expression vectors for expressing the polypeptides of VHg-VLh obtained in (ii) of Example 19 and of VHh-VLg obtained in (iv) of Example 19 were treated in individual systems in a step of expressing and purifying a protein to be described later, and

20 were obtained as polypeptide chains VHg-VLh and VHh-VLg.

1) Transformation

The above expression vectors were used to transform different 40 µL of BL21 (DE3) competent

25 cell solutions. The transformation was performed under such a condition that heat shock was performed in ice → 42°C × 90 sec → in ice. 750 µL of LB medium

were added to the BL21 solution transformed by heat
shock, and the whole was cultured with shaking for 1
hour at 37°C. After that, centrifugation was
performed at 6,000 rpm × 5 min, and 650 µL of the
culture supernatant were discarded. The remaining
culture supernatant and a cell fraction as a
precipitate were stirred and inoculated on an LB/amp.
plate, and the whole was left standing at 37°C
overnight.

2) Preculture

A colony on the plate was selected at random
and was cultured with shaking at 28°C overnight with
3.0 mL of an LB/amp. medium.

3) Main culture

The preculture solution was subcultured in 750
ML of a 2 × YT medium, and the culture was continued
at 28°C. When OD_{600} exceeded 0.8, IPTG was added to
have a final concentration of 1 mM, and culture was
performed at 28°C overnight.

4) Purification

A target polypeptide chain was purified from an
insoluble granule fraction through the following step.
(i) Collection of insoluble granule

The culture solution obtained in 3) above was
centrifuged at 6,000 rpm × 30 min to obtain a
precipitate as a bacterial fraction. The resultant
was suspended in 15 ml of a Tris solution (20 mM

Tris/500 mM NaCl) in ice. The resultant suspension
was homogenized with a French press to obtain a
bacterial homogenized solution. Next, the bacterial
homogenized solution was centrifuged at 12,000 rpm ×
5   15 min, and the supernatant was removed to obtain a
precipitate as an insoluble granule fraction.
(ii) Solubilization of insoluble granule fraction
        The insoluble fraction obtained in (i) above
was added with and immersed in 10 mL of a 6 M
10   guanidine hydrochloride/Tris solution overnight.
Next, the resultant was centrifuged at 12,000 rpm ×
10 min to obtain a supernatant as a solubilized
solution.
(iii) Metal chelate column
15   A His-Bind (manufactured by Novagen) was used
as a metal chelate column carrier. Column adjustment,
sample loading, and a washing step were performed at
room temperature (20°C) in accordance with the method
recommended by the supplier. Elution of a His tag-
20   fused polypeptide as a target was performed in a 60
mM imidazole/Tris solution. SDS-PAGE (acrylamide
15%) of the eluent confirmed that the eluent had a
single band and was purified.
(iv) Dialysis
25   The eluent was dialyzed by using a 6 M
guanidine hydrochloride/Tris solution as an external
solution at 4°C to remove imidazole in the eluent,

thereby obtaining the above each polypeptide chain
solution.

(v) Refolding

In the same manner as that described above, the
5 polypeptide chain solutions of VHg-VLh and VHh-VLg
were separately dialyzed (4°C) according to the
following steps by using dehydrochlorinated guanidine
to perform refolding of the protein.

(a) A sample having a concentration of 7.5 µM (volume
10 after dilution of 10 ml) was prepared by using a 6 M
guanidine hydrochloride/Tris solution on the basis of
the molar absorbance coefficient and ΔO.D. (280 nm-
320 nm) of each polypeptide chain. Next, β-
mercaptoethanol (reductant) was added to have a final
15 concentration of 375 µM (50-fold protein
concentration), followed by reduction at room
temperature in a dark room for 4 hours. The sample
solution was charged into a dialysis bag (NWCO:
14,000) and provided as a sample for dialysis.

20 (b) The sample for dialysis was immersed in a 6 M
guanidine hydrochloride/Tris solution as an external
solution, and was dialyzed for 6 hours while being
gently stirred.

(c) The guanidine hydrochloride solution of the
25 external solution was reduced to 3 M and then to 2 M
in a stepwise manner. The sample was dialyzed at
each concentration of the external solution for 6

hours.

(d) The Tris solution was added with oxidized
glutathione (GSSG) to have a final concentration of
375 μM and with L-Arg to have a final concentration
5   of 0.4 M), and then was added with the 2 M dialysis
external solution in (c) above to have a guanidine
hydrochloride concentration of 1 M. The sample was
·dialyzed in a solution whose pH had been adjusted to
8.0 (4°C) with NaOH for 12 hours while being gently
10 stirred.

(e) An L-Arg Tris solution having a guanidine
hydrochloride concentration of 0.5 M was prepared by
the same operation as that of (d), and dialysis was
performed for an additional 12 hours.
15 (f) Finally, dialysis was performed for 12 hours in a
Tris solution.

(g) After the completion of the dialysis,
centrifugation was performed at 10,000 rpm for about
20 min to separate an aggregation and a supernatant.
20 (vi) Purification of dimerized fraction

The respective 5 μM polypeptide (VHg-VLh,
VHh-VLg) solutions obtained in (v) above were mixed and
left standing at 4°C overnight. Next, a fraction
corresponding to 60 kDa (about 18 minutes from the
25 injection) dimerized in a Sephadex 75 column (column:
buffer 20 mM Tris, 500 mM NaCl, flow rate: 1 ml/min)
was obtained. This was provided as a sample for SPR

measurement.

Example 21 (Evaluation of binding affinity for gold through SPR measurement)

The binding affinity of the dimerized protein fraction obtained in Example 20 for gold was measured by means of SPR. A BIAcore 2000 (manufactured by BIAcore) was used as an SPR measuring device. A gold-deposited glass substrate SIA-kit Au of the same company was used as a gold substrate (to which the fraction was to be bound). Measurement was performed under the following conditions. 500 nM (calculated from the absorbance as described above) of the dimerized protein fraction obtained in Example 15 were used as a sample.

Running buffer: 0.1% Tween 20/Tris solution : TBST
Temperature: 25°C
Flow rate: 20 µL/min
Sample injection amount: 40 µL

A binding curve showing the binding affinity for gold was obtained (Fig. 18).

Example 22 (Evaluation of binding affinity to HEL through SPR measurement)

Subsequent to the sample subjected to SPR evaluation of binding affinity for gold in Example 21, a 1 µM HEL solution was continuously injected to measure binding affinity of the gold-bound dimerized protein fraction to HEL by means of SPR. A binding

curve showing the binding affinity to HEL was
obtained (Fig. 18).

Example 23 (Confirmation of Au specificity)

The gold substrate of an SPR chip obtained in

5   Example 22 to which the gold-binding protein and HEL
were bound was used to subsequently perform the
following experiment. The gold substrate described
above was injected with a 1 µM anti-HEL antibody/PBST
solution. After that, the substrate was washed with

10 PBST. Furthermore, the substrate was injected with a
1 µM rhodopsin-binding anti-IGg antibody/PBST
solution. After that, the substrate was washed with
PBST. The SPR chip described above was removed from
an SPR device, and the substrate was observed with a

15 fluorescence microscope. As a result, fluorescence
was observed on a flow path of SPR on the gold
substrate.

Comparative Example 2

The same operation as that of Example 23 was

20 performed by using an unused gold-deposited substrate.
As a result, no fluorescence was observed on the gold
substrate.

Example 24

A rewound polypeptide chain VHg-VLh obtained

25 through the steps as those of (i) to (v) in Example
20 was prepared, and a 5 µM VHg-VLh/Tris solution was
obtained.

Example 25

The VHg-VLh/Tris solution obtained in Example 24 was diluted with a Tris solution to 500 nM, and the binding affinity to the gold substrate was

5   evaluated in the same manner as in Example 20. Furthermore, a 1 μM HEL solution was continuously injected to measure the binding affinity of the gold-bound dimerized protein fraction to HEL. A binding curve showing binding affinity for gold and HEL was

10   obtained.

Example 26

In Example 25, a 0.5 μM anti-HEL antibody VL/Tris solution was allowed to coexist, and the whole was left standing overnight to prepare a sample.

15   Example 27

The sample prepared in Example 26 was evaluated for binding affinity to the gold substrate by means of SPR in the same manner as in Example 15. Furthermore, in the same manner as in Example 16, a 1

20   μM HEL was continuously injected to measure binding affinity to the sample by means of SPR. A binding curve showing the binding affinity was obtained.

Example 28 (Preparation of HEL detecting immunochromatography device)

25   An immunochromatography device for detecting HEL was prepared as an example of detecting method of the present invention.

1) Preparation of porous carrier for immunochromatography on which anti-HEL antibody is immobilized

A nitrocellulose sheet (BAS-85, manufactured by
5   Schleicher & Schuell) was cut into a piece measuring 5 mm × 30 mm. A 0.5 mg/mL of an anti-HEL antibody solution (manufactured by Nippon Biotest Labo) was linearly applied at a position 10 mm away from the end of the piece in an amount of 0.5 mg/mL, to
10 thereby prepare a detection site. The sheet was left standing at room temperature for 2 hours to dry the liquid, whereby the antibody was fixed as a sheet. The sheet was shaken for 2 hours in a 1% skim milk (manufactured by Difco)/PBST solution and blocked.
15 After that, the sheet was left standing at room temperature to prepare a carrier for immunochromatography.

2) Preparation of gold-labeled anti-HEL antibody fragment and hold-back carrier

20   (i) Preparation of gold-labeled anti-HEL antibody fragment

The fragment of this title was prepared according to the following step by using the gold/HEL bispecific antibody fragments prepared in Example 20.

25   A dispersion of gold fine particles (particle size 50 nm, manufactured by Tanaka Kikinzoku) was added and sufficiently mixed with the gold/HEL

bispecific antibody fragments prepared in Example 20,
and a reaction was performed at room temperature for
3 hours. The resultant was centrifuged at 12,000 rpm
for 5 min in order to remove unreacted gold/HEL
5   bispecific antibody fragments, and then the
supernatant was removed to obtain a precipitate. The
resultant precipitate was suspended in 1.0 mL of PBST.
Furthermore, centrifugation was performed under the
above conditions. The resultant precipitate was
10   suspended in 1.0 mL of 1% BSA/PBST.

    (ii) Preparation of gold-labeled anti-HEL antibody
fragment-holding carrier

    A Bemliese non-woven fabric (manufactured by
Asahi Kasei Corporation) measuring 5 mm × 5 mm was
15   impregnated with 5 µL of the gold-labeled anti-HEL
antibody fragment solution and 5 µL of a 10% aqueous
solution of various bases, and was air-dried to
prepare a gold-labeled anti-HEL antibody fragment-
holding carrier.

20   3) Preparation of test piece-type
immunochromatography device

    The gold-labeled anti-HEL antibody fragment-
holding carrier prepared in 2) (ii) above was allowed
to overlap the carrier for immunochromatography
25   prepared in 1) above up to a position 2.5 mm away
from one end of the carrier for immunochromatography.
Furthermore, a carrier for soaking a liquid sample

(filter paper No. 526, manufactured by Advantec Toyo)
was allowed to overlap the antibody fragment-holding
carrier. In addition, a carrier for an excess
sample-soaking portion (filter paper No. 526) was

5   allowed to overlap the carrier for
immunochromatography up to a position 5 mm away from
the other end of the carrier for immunochromatography.
Finally, a tape was attached to the rear side to
stabilize the entirety, whereby a test piece-type

10 immunochromatography device was prepared. A test
using a standard HEL solution confirmed that an anti-
HEL antibody-immobilized portion presented a red
color.

      Example 29 (Preparation of electrical measuring

15 instrument)

      An example of a method of detecting a protein
using a gold electrode is shown.
1) 2 gold electrodes were placed on a glass substrate.
The distance between the electrodes was set to 20 μm.

20 20 μL of a 0.1% poly-L-lysin (Sigma-Aldrich) aqueous
solution were dropped onto a space between the
electrodes, and the whole was left standing for 3
hours.

      Next, the substrate was washed with water.

25 After that, the substrate was washed with ethanol 3
times and dried.

      2) Next, an anti-HEL polyclonal antibody (ROCKLAND)

was immobilized on the glass substrate obtained in

(a) above on the basis of Proteomics, 3, pp254 (2003).

3) The PBST solution of the gold/HEL bispecific

antibody fragment prepared in Example 20 and 20-nm

5   gold nanoparticles (manufactured by Tanaka Kikinzoku)

were allowed to react with each other (PBST solution

for comparison).

4) The anti-HEL antibody-immobilized substrate

obtained in 1) was added with a 1 µM HEL/PBS solution.

10 5) Next, the product obtained in 3) was added with

the gold-binding protein solution obtained in 2).

6) The substrate obtained in 4) was washed with a PBS

solution 3 times. After that, the substrate was

immersed in a silver enhancer solution (Sigma—

15 Chemical) for 5 min, followed by washing with water.

A reduction in electrical resistance between the

electrodes as compared to that of only PBST as a

comparative sample was confirmed.

Example 30 (Preparation of vector for

20 expressing VHg-VHh protein in yeast)

(1) Preparation of NheI-VHh-SacII fragments

VHh fragments having NheI/SacII at its terminals were

prepared by PCR by using a pHGold prepared in Example

19 as a template.

25   The following primers were used.

NheI-VHh_f (SEQ ID No: 131)

NNNNNGCTAGCCAGGTGCAGTTGGTGGAGTCT

VHh-sacII_r (SEQ ID No: 132)

NNNNNCCCGCGGATGAGGAGACGGTGACCAGGGTT

The above primers were used to perform PCR of
pfu-turbo in accordance with a method recommended by
5   a supplier. The resultant PCR reaction solution was
subjected to agarose gel (2%) electrophoresis to
confirm that about 350 bp of fragments were obtained.
(2) Preparation of VHg-VHh DNA fragments
The VHh fragments obtained in (1) above were
10   introduced into a VLh portion of pGHEL prepared in
Example 19.
The PCR fragments and pGHEL obtained in (1)
were cleaved with NheI/SacII (each manufactured by
TAKARA BIO INC.). A restriction enzyme reaction was
15   performed in accordance with a method recommended by
one skilled in the art. The resultant reaction
solutions were subjected to agarose electrophoresis,
followed by gel purification (PCR fragment: agarose
2%, pGHEL: agarose 1%). The above-described gel
20   purification kit was used for the gel purification.
It was confirmed that the PCR fragments
obtained after the restriction enzyme reaction as
described above had about 350 bp of PCR fragments and
3,000 bp of plasmid fragments, and a novel plasmid
25   were obtained hereinafter in the same manner as in
Example 19.
The base sequence of the resultant plasmids was

determined by means of a sequencer to confirm that
the base sequence was a target base sequence (the
plasmid was defined as pHgHh).

(3) Insertion of yeast expression plasmids

5   PCR was performed in accordance with the same
method that recommended by a supplier.

pPCIZαA (Invitrogen) was used as an yeast
(Pichia pastris) expression plasmid. Target gene was
introduced by using EcoRI and SacII at multicloning

10   sites of the plasmid.

The gene to be introduced was prepared by PCR
by using pHgHh obtained in (2) as a template.
As a primer,
7s4-fW-EcoRls (SEQ ID No: 133)

15   AAGCTGAATTCCAGGTGCAGTTGGTGGAGTCT
HELVH-SacII-r (SEQ ID No: 134)
NNNNNCCGCGGAGACGGTGACGAGGGT
The resultant PCR fragments and the pPCIZαA were
sequentially cleaved with EcoRI and SacII, and each

20   target fragment was obtained in the same manner as
that described above through gel purification.

Ligation was performed in accordance with the
method described above. A ligation reaction solution
was transformed in the same manner as that described

25   above and inoculated on an agar plate. The agar
plate in this case was such that tryptophan 10
. g/yeast extract 5 g/NaCl 5 g/agar 15 g/L were added

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with Zeocin to have a concentration of 25 μg/L. A

colony selected by this condition was cultured in a

liquid medium (tryptophan 10 g/yeast extract 5 g/NaCl

15 g, Zeocin 25 μg/L) at 37°C overnight. After the

5   collection of plasmids, a sequence was confirmed by

using a sequencer to obtain a plasmid expressing VHg-

VHh : VH_gold-linker (GGGGS)-VH_HEL as an object of

this example (provided as pPCIZ-αHH).

Example 31 (Expression/purification of VHg-VHh

10   protein)

The expression of VHg-VHh was performed by

using an Easy Select Pichia Expression Kit Ver. G

(Invitrogen). The preparation of a transformant and

the preparation and purification of the protein

15   (metal chelate column) were each performed in

accordance with a method recommended by one skilled

in the art. 5 mL of a 1 M imidazole eluted fraction

obtained by a metal chelate column were dialyzed by

using a Tris buffer (20 mM Tris/200 mM NaCl, 1 mM

20   EGTA: pH 7.9) as an external solution at 4°C. The

external solution was changed every 6 hours and a

total of 3 times.

Subsequently, by using a Sephadex 75 (Amasham

Bioscience), purification based on gel filtration was

25   performed at 4°C (buffer condition: 50 mM Tris-HCl,

200 mM NaCl, 1 mM EDTA, pH 8.0, flow rate: 0.7

mL/min). After the resultant fraction had been

concentrated, the same western Blotting as that

described above was performed by using SDS-PAGE

(acrylamide 17.5%) and HRP-fused anti-His antibody.

Thus, a fraction of the target protein was specified

5   and purified into a single band. A peak suggesting

that the fraction was a monomer protein of about 25

kDa was fractionated, followed by the following

evaluation (FIG. 19).

Example 32 (Evaluation of binding affinity for

10   gold by means of SPR)

The binding affinity of the protein fraction

obtained in Example 31 for gold was measured by means

of SPR. A BIAcore 2000 (manufactured by BIAcore) was

used as an SPR measuring device. A gold-deposited

15   glass substrate SIA-kit Au of the same company was

used as a gold substrate to which the fraction was to

be bound. Measurement was performed under the

following conditions. 500 nM (calculated from the

absorbance as described above) of the protein

20   fraction obtained in Example 20 were used as a sample.

The conditions of this example were the same as those

Example 21. A binding curve showing binding affinity

for gold was obtained (Fig. 20).

Example 33 (Preparation of VHg-VHh protein

25   variant -1)

A QC kit (manufactured by STRATAGENE) was used

with the plasmid pPCIZ-α7s4 obtained in Example 30 as

a template to obtain a VHg variant (SEQ ID No: 135 or

136) to serve as V37L, G44E, and L45R of the gold-

binding VH of Example 30. The following primers were

used to obtain a target plasmid through 3 times of

5   operation. Mutation was sequentially inserted for

each operation into one site.

PCR primer for inserting mutation to a first site

V37F-f (SEQ ID No: 137)

TTACTGGATCAACTGGTTCCGCCAGATGCCCGG

10   V37F-r (SEQ ID No: 138)

CCGGGCATCTGGCGGAACCAGTTGATCCAGTAA

PCR primer for introducing mutation to a second site

G44E-f (SEQ ID No: 139)

15   CAGATGCCCGGCAAAGAACTGGAATGGATGGGG

G44E-r (SEQ ID No: 140)

CCCCATCCATTCCAGTTCTTTGCCGGGCATCTG

PCR primer for introducing mutation to a third site

20   L45F-f (SEQ ID No: 141)

GCCCGGCAAAGAAAGGGAATGGATGGGGATG

L45F-r (SEQ ID No: 142)

CATCCCCATCCATTCCCTTTCTTTGCCGGGC

Insertion of mutation was confirmed by a

25   sequence. The procedure from the transformation to

the expression of a protein was the same as that of

Example 31. The following evaluation was performed

by using a peak of a monomer protein of about 25 kDa.

Example 34 (Evaluation of binding affinity for gold by means of SPR)

The binding affinity of the protein fraction

5   obtained in Example 33 for gold was measured by means of SPR. A BIAcore 2000 (manufactured by BIAcore) was used as an SPR measuring device. A gold-deposited glass substrate SIA-kit Au of the same company was used as a gold substrate to which the fraction was to

10   be bound. Measurement was performed under the following conditions. 500 nM (calculated from the absorbance as described above) of the protein fraction obtained in Example 33 were used as a sample. The conditions of this example were the same as those

15   Example 21. A binding curve showing binding affinity for gold was obtained (Fig. 21).

Example 35 (Preparation of VHg-HEL scFv protein variant)

Substitution was performed in such a manner

20   that a protein fused with HEL-binding scFv (SEQ ID No: 143 or 144) instead of HEL-binding VH of Example 31 was obtained. DNA fragments encoding HEL-binding scFv was obtained by PCR with a plasmid to which an HEL-binding scFv coding gene was introduced, show in

25   Journal of Biological chemistry, 2003, 279, pp8979, as a template. PCR was performed in accordance with the same method as that recommended by one skilled in

the art, and the following primers were used.

scFv-f (SEQ ID No: 145)

NNNNCCATGCCCGATATCGTCCTGACCCAG

scFv-r (SEQ ID No: 146)

5   AGCTACCGCGGAGACGGTGACGAGGGT

The procedure subsequent to a restriction

enzyme reaction was the same as that of Example 30,

whereby a target plasmid was obtained.

It was confirmed by using a sequence that the

10   resultant plasmid had a target gene sequence. The

procedure from the transformation to the expression

of a protein was the same as that of Example 31.

Thus, a monomer protein of about 39 kDa was obtained.

Example 36 (Evaluation of double-binding

15   affinity for gold and HEL by means of SPR)

The binding affinity of the protein fraction

obtained in Example 35 for gold was measured by means

of SPR. A BIAcore 2000 (manufactured by BIAcore) was

used as an SPR measuring device. A gold-deposited

20   glass substrate SIA-kit Au of the same company was

used as a gold substrate to which the fraction was to

be bound. Measurement was performed under the

following conditions. 500 nM (calculated from the

absorbance as described above) of the protein

25   fraction obtained in Example 31 were used as a sample.

The conditions of this example were the same as those

Example 21 and 22. A binding curve showing binding

affinity for gold was obtained (Fig. 22).

　　　Example 37 (Preparation of plasmid for expressing VHg variant-VLh)

　　　A DNA sequence represented by SEQ ID No: 147

5　and 148 were introduced into a gold-binding VH code sequence of the plasmid for expression (pGHEL) obtained in Example 19. An insertion method involved the use of the Quick Change kit described above (Stratagene) with the pGHEL as a template above. It

10　was confirmed that the resultant plasmid had a target sequence. Next, the resultant plasmid was used to perform the expression and purification of a protein and the dimerization with VHh-VLg in the same manner as in Example 20.

15　　　A protein fraction having a molecular weight of about 50 kDa was fractionated by using a Sephadex G75 (Fig. 23).

A14P-f (SEQ ID No: 147)

GAGCAGAGGTGAAAAAGCCAGGGGAGTCTCTGAAG

20　A14P-r (SEQ ID No: 148)

CTTCAGAGACTCCCCTGGCTTTTTCACCTCTGCTC

　　　Example 38 (Evaluation of double-binding affinity for gold and HEL by means of SPR)

　　　The binding affinity of the protein fraction

25　obtained in Example 37 for gold was measured by means of SPR. A BIAcore 2000 (manufactured by BIAcore) was used as an SPR measuring device. A gold-deposited

glass substrate SIA-kit Au of the same company was

used as a gold substrate to which the fraction was to

be bound. Measurement was performed under the

following conditions. 500 nM (calculated from the

5   absorbance as described above) of the protein

fraction obtained in Example 37 were used as a sample.

The conditions of this example were the same as those

Example 21. A binding curve showing binding affinity

for gold was obtained (Fig. 24).

10   Example 39 (Preparation of plasmid expressing

VHg-VLg tetramer)

pPCIZ-αVHg^2 was prepared in the same manner as

in Example 30 except that VHh of Example 30 was

changed to VHg coding gene fragments.

15   pRA2-7s4 obtained in Example 10 was used as a

template for preparing a DNA fragments encoding the

VHg to be used in the above operation, and the

following primers

VHg-f (SEQ ID No: 149)

20 NNNNNGCTAGC GGCGGGGGCGGTAGC CAGGTGCAGTTGGTGGAGTCT

VHg-r (SEQ ID No: 150)

NNNNNCCGCGGATGAGGAGACGGTGACCAGGGTT

were used as primers

It was confirmed by a sequence that a target

25 plasmid was obtained.

Example 40 (Preparation of VHg-VLg protein)

A target protein was purified by the same

procedure as that of Example 31. A protein composed
of a dimer of VHg-VHg having a molecular weight of
about 25 kDa was purified (Fig. 25).

Example 41 (Preparation of plasmid expressing
VHg-VLg tetramer)

pPCIZ-αVHg^4 was prepared in the same manner as
in Example 30 except that the linker GGGGS for
linking VHg-VHg of Example 39 was changed to GS.

pRA2-7s4 obtained in Example 10 was used as a
template for preparing a DNA fragment encoding the
VHg to be used in the above operation, and the
following primers
VHg4-f (SEQ ID No: 151)
NNNNNGCTAGC GGCAGC CAGGTGCAGTTGGTGGAGTCT
VHg4-r (SEQ ID No: 152)
NNNNNCCGCGGATGAGGAGACGGTGACCAGGGTT
were used as primers.

It was confirmed by a sequence that a target
plasmid was obtained.

Example 42 (Gold fine particles agglutination
reaction)

In a 500 μM/PBST solution of each of the
proteins obtained in Examples 40 and 44, gold fine
particles (20 nmφ: manufactured by Tanaka Kikinzoku)
were incubated at room temperature. The
agglutination of the gold fine particles was observed
regardless of which protein was used. In addition, a

spectrum (λmax) of a mixture of gold fine particles/a

protein was observed to change with time, and a half

band width was observed to expand. A result

suggesting that the distance between gold fine

5   particles was shortened was obtained (Fig. 26).

Comparative Example 3

The same operation as example 36 was performed

by using 500 nM BSA instead of 500 nM HEL. Binding to

BSA was not confirmed. It was shown that the protein

10   for gold obtained in example 35 binds to HEL in a

specific manner (Fig. 27).

Comparative Example 4

Binding ability of proteins immobilized

directly on gold substrate was evaluated by anti-HEL

15   antibody (manufactured by Rockland) using SPR

measurement.

(1)   Preparing 10 μM HEL in PBS

(2)   Injecting 100 μl of the solution obtained (1)

at 1 μl/min. The signal of absorbed antibody was 1907

20   R. U.

(3)   1% casein in PBS was injected in the same

manner as (2)

(4)   Further, 40 μl of 1% casein in PBS was injected

at 20 μl/ml and confirm that the gold substrate was

25   blocked.

(5)   Consequently, injecting 40 μl of 1 μM at

20μl/min and obtaining the result shown in Fig. 28.

As the result, the signal of bond HEL molecule
was 11 R. U., while that of antibody immobilized on
gold substrate was 1907 R. U. Considering of

5   configuration at immobilization and assuming that a
single antibody molecule captured one antigen on a
substrate, the rate of antibodies captured target
substances was calculated to be about 6%.

In the other hand, it was shown that 20 to 40 %

10 of proteins in the present invention captured
antigens by examples indicated above. Moreover, any
special reagents or process was not required for the
immobilization of proteins that are capturing
molecules of sensors in the examples indicated above,

15 implying that the immobilization method of the
present invention is superior to conventional
physical absorption.

INDUSTRIAL APPLICABILITY

20   The present invention provides: a gold-binding
protein having one or more binding sites to gold and
a binding site to a specific substance; a structure
including a gold substrate on which the gold-binding
protein is immobilized; and a detection device using

25 the same.

In a detection device composed of a structure
on which a gold-binding protein obtained by applying

the present invention is immobilized, the protein is

immobilized at a binding site specifically

recognizing gold serving as a substrate. Therefore,

the binding site of the protein recognizing a

5   specific substance (target substance) is not

immobilized on the substrate, and the protein is

oriented while a distance from the substrate is

ensured. As a result, a target substance binding

site minimizes the influence of the substrate on its

10   binding capacity, whereby the protein is immobilized

on the substrate with efficiency and high orientation.

That is, it is suggested that the present

invention can be utilized for improving the

performance of products applying the functions of

15   various biological substances which immobilize

organic substances such as biological substances on

substrate surfaces to utilize various physiological

functions of the organic substances, the products

being typified by a biosensor and a bioreactor.

20   Meanwhile, the present invention provides a

connecting member for labeling a target substance,

including: one or more sites binding to the target

substance; and one or more sites binding to the

labeled substance, characterized in that the

25   respective binding sites bind to a substance to be

bound independently from each other. The application

of the present invention enables a target protein to

be labeled without the use of a conventional chemical crosslinking method. As a result, influences on the binding affinities of various proteins to target substances, the influences being of concern at the

5   time of labeling, can be minimized, and production efficiency can be increased. That is, the present invention can be utilized for improving the performance of products applying the functions of various biological substances, such as biosensor,

10   which immobilize connecting members on substrate surface, and utilize various physiological functions of the connecting members.

15   This application claims priority from Japanese Patent Application No. 2004-108388 filed March 31, 2004, which is hereby incorporated by reference herein.

CLAIMS

1. A protein having affinity for gold,
comprising at least a portion of an antibody.

5   2. A protein according to claim 1, comprising a
gold-binding site containing at least a portion of
the antibody.

3. A protein according to claim 2, wherein the
gold-binding site comprises at least one selected
10 from F(ab')2, Fab', Fab, and a portion thereof.

4. A protein according to claim 2, wherein the
gold-binding site comprises at least one selected
from an antibody variable region (Fv) and a portion
thereof.

15   5. A protein according to claim 4, wherein the
gold-binding site comprises at least one selected
from:

(1) an antibody heavy chain variable region
(VH), a variant thereof, and a portion thereof; and

20   (2) an antibody light chain variable region
(VL), a variant thereof, and a portion thereof.

6. A protein according to claim 5, wherein the
antibody heavy chain variable region (VH) comprises
at least one of amino acid sequences of SEQ ID NOs.:
25 1 to 48.

7. A protein according to claim 5, wherein the
variant of the antibody heavy chain variable region

(VH) contains one or more of amino acid sequences
with deletion, substitution, or addition of one or
several amino acids in the amino acid sequences of
SEQ ID NOs.: 1 to 48, and has affinity for gold.

5   8. A protein according to claim 5, wherein the
antibody light chain variable region (VL) comprises
at least one of amino acid sequences of SEQ ID NOs.:
49 to 57.

         9. A protein according to claim 5, wherein the
10 variant of the antibody light chain variable region
(VL) contains one or more of amino acid sequences
with deletion, substitution, or addition of one or
several amino acids in the amino acid sequences of
SEQ ID NOs.: 49 to 57, and has affinity for gold.

15   10. A protein according to claim 1, wherein a
dissociation constant is 10^{-6} M or less.

         11. A gold-binding complex protein, comprising:

         (1) a first domain containing a protein having
affinity for gold according to any one of claims 1 to

20 10; and

         (2) a second domain containing a protein having
a binding site to a certain substance.

         12. A gold-binding complex protein according to
claim 11, further comprising at least one of a third

25 domain forming a complex with the first domain and a
fourth domain forming a complex with the second
domain.

13. A gold-binding complex protein according to claim 12, wherein the third domain has affinity for gold.

14. A gold-binding complex protein according to
5 claim 12, comprising any one of the following structures (1) to (7):

(1) the first domain and the second domain form one polypeptide chain;

(2) the first domain and the second domain are
10 bound via one or more amino acids;

(3) the third domain and the fourth domain form one polypeptide chain;

(4) the third domain and the fourth domain are bound via one or more amino acids;

15 (5) at least the first domain, second domain, and third domain form one polypeptide chain;

(6) at least the first domain, second domain, and fourth domain form one polypeptide chain; and

(7) all of the first domain to the fourth
20 domain form one polypeptide chain.

15. A structure, comprising a substrate and a protein, wherein the substrate contains gold in at least a portion of its surface and the protein comprises a gold-binding complex protein according to
25 claim 11.

16. A structure, comprising a substrate and a protein, wherein the substrate contains gold in at

least a portion of its surface and the protein

comprises a gold-binding complex protein according to

claim 12.

17. A structure according to claim 15, wherein

the second domain has affinity for a target substance.

18. A structure according to claim 16, wherein

at least one of the second domain and the fourth

domain has affinity for a target substance.

19. A nucleic acid encoding a gold-binding

complex protein according to claim 11.

20. A nucleic acid encoding a gold-binding

complex protein according to claim 12.

21. A nucleic acid encoding a gold-binding

complex protein according to claim 13.

22. A nucleic acid encoding a gold-binding

complex protein according to claim 14.

23. A vector comprising a nucleic acid

according to claim 19.

24. A vector comprising a nucleic acid

according to claim 20.

25. A vector comprising a nucleic acid

according to claim 21.

26. A vector comprising a nucleic acid

according to claim 22.

27. A method of producing a structure according

to claim 15, comprising the step of:

(1) preparing the substrate;

(2) producing the gold-binding complex protein; and

(3) arranging the gold-binding complex protein on the substrate.

28. A method of producing a structure according to claim 16, comprising the step of:

(1) preparing the substrate;

(2) producing the gold-binding complex protein; and

(3) arranging the gold-binding complex protein on the substrate.

29. A method of producing a structure according to claim 27, wherein the production of the gold-binding complex protein is performed by introducing a vector containing a nucleic acid encoding the gold-binding complex protein into a host cell to thereby express a polypeptide chain containing the gold-binding complex protein.

30. A kit for detecting a target substance, comprising a substrate and a gold-binding complex protein for forming a structure according to claim 15, and detection means for detecting binding of the target substance to the structure.

31. A kit for detecting a target substance, comprising a substrate and a gold-binding complex protein for forming a structure according to claim 16, and detection means for detecting binding of the

target substance to the structure.

32. A connecting member for labeling a target substance with a labeling agent, comprising:

one or more sites binding to the target

5   substance and one or more sites binding to the labeling agent, wherein:

the site binding to the target substance and the site binding to the labeling agent each independently bind to a substance to be bound; and

10   at least one of the site binding to the labeling agent and the site binding the target substance contains a protein according to any one of claims 1 to 11.

33. A connecting member according to claim 32,

15   wherein the labeling agent or the target substance has at least one or more of a metal, a metal oxide, and a semiconductor compound each containing gold.

34. A method of detecting a target substance, in which the target substance is bound and labeled

20   with a labeling agent to detect the target substance bound with the labeling agent, comprising the step of:

binding the labeling agent to the target substance via a connecting member according to claim

25   32.

35. A kit for detecting a target substance, comprising: a labeling agent containing gold; a

connecting member according to claim 31; and
detection means for detecting a state in which the
labeling agent is bound to the target substance via
the connecting member.

FIG. 1
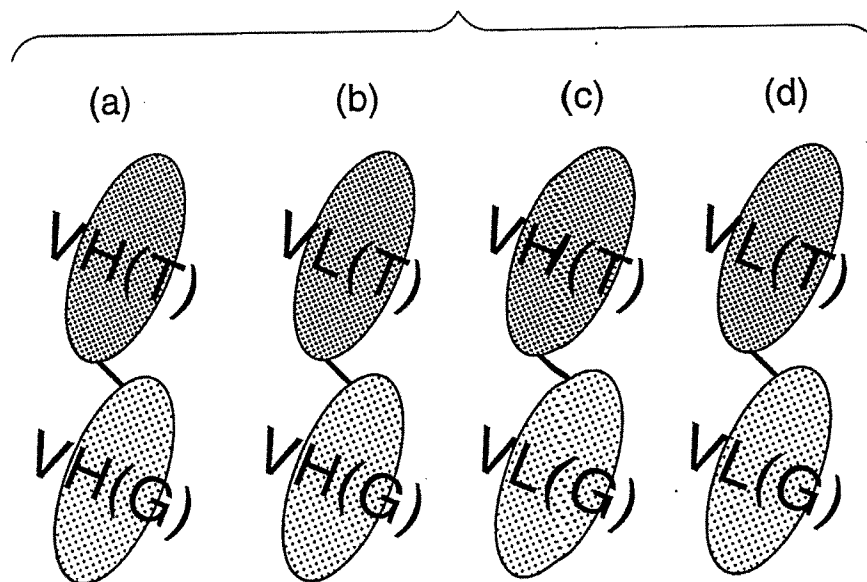
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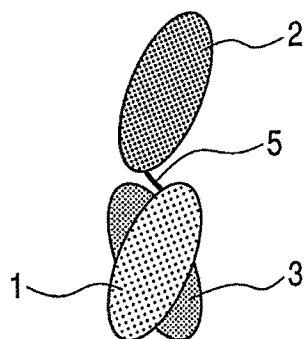
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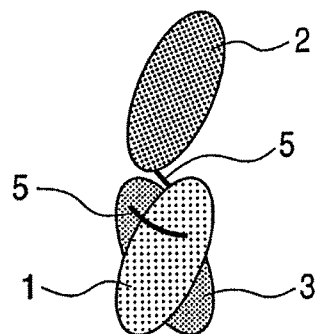
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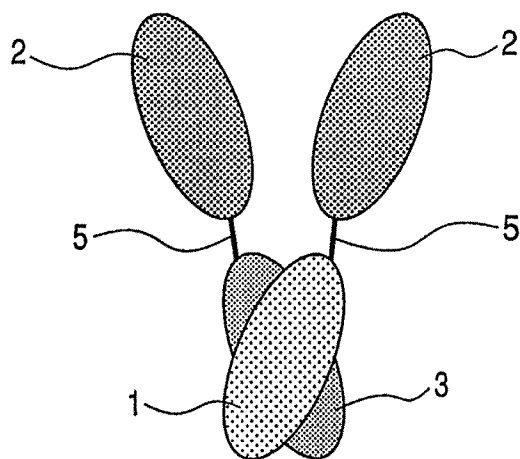
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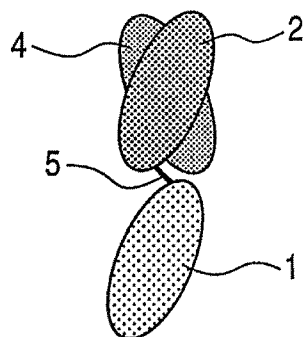
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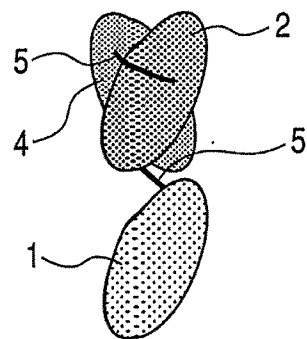
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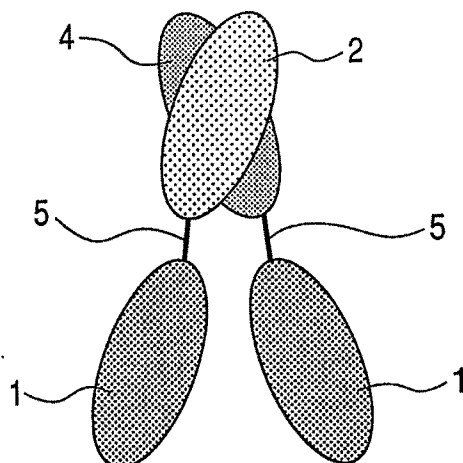
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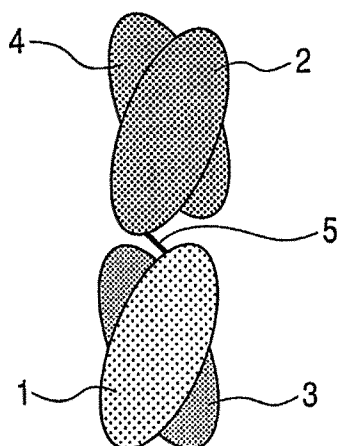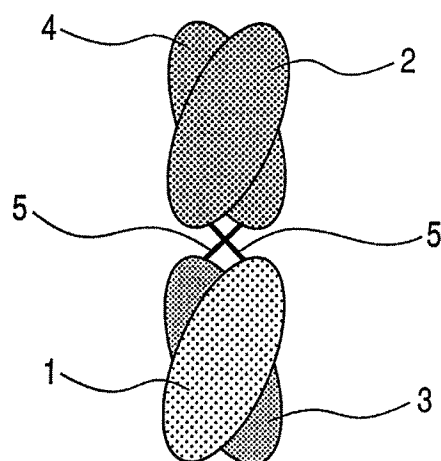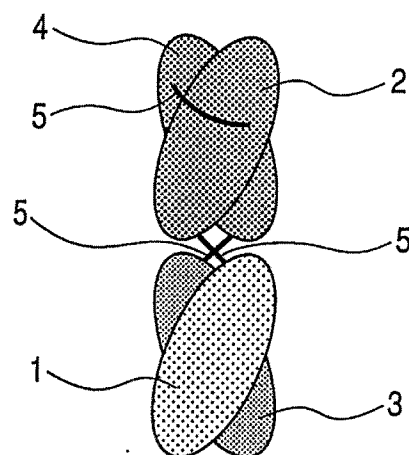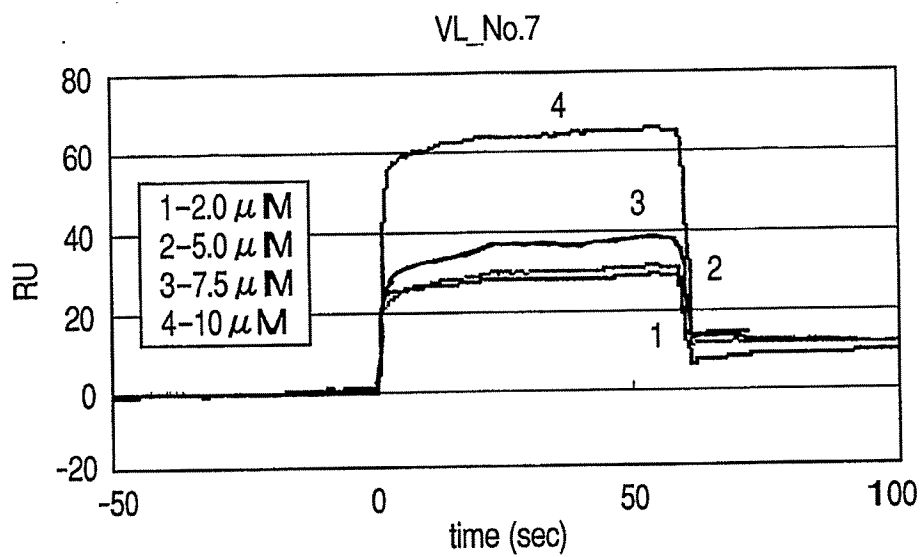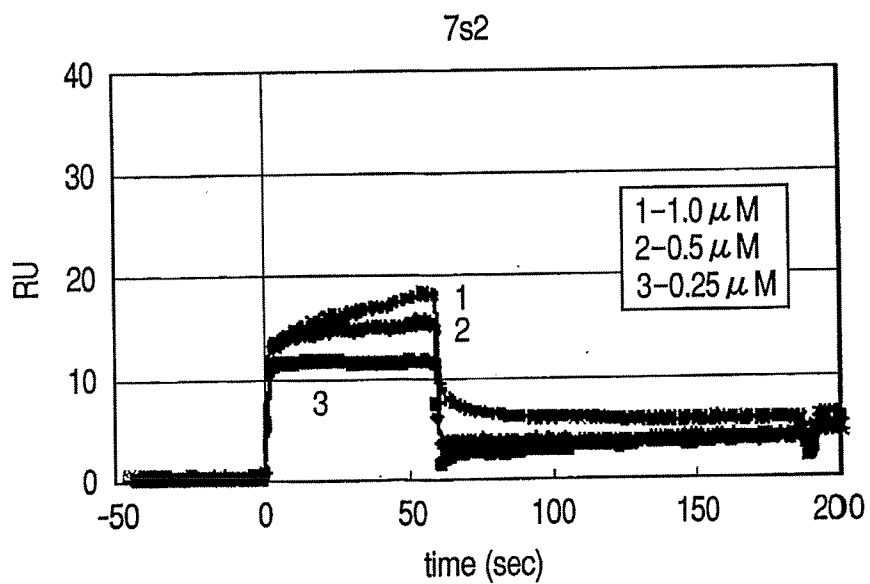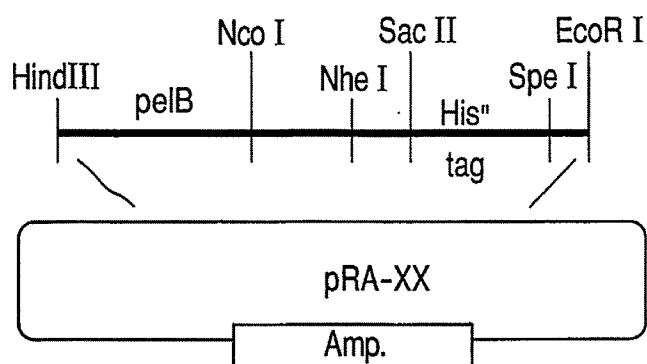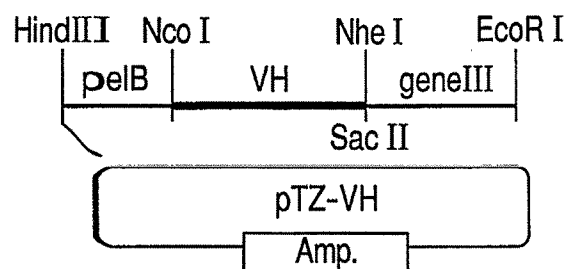
FIG. 7

FIG. 8

FIG. 9

VL_No.7

FIG. 10

7s2

FIG. 11

FIG. 12A

FIG. 12B
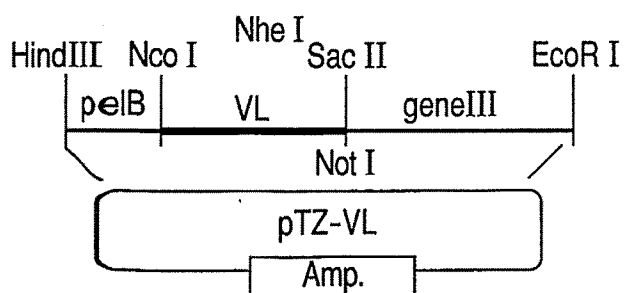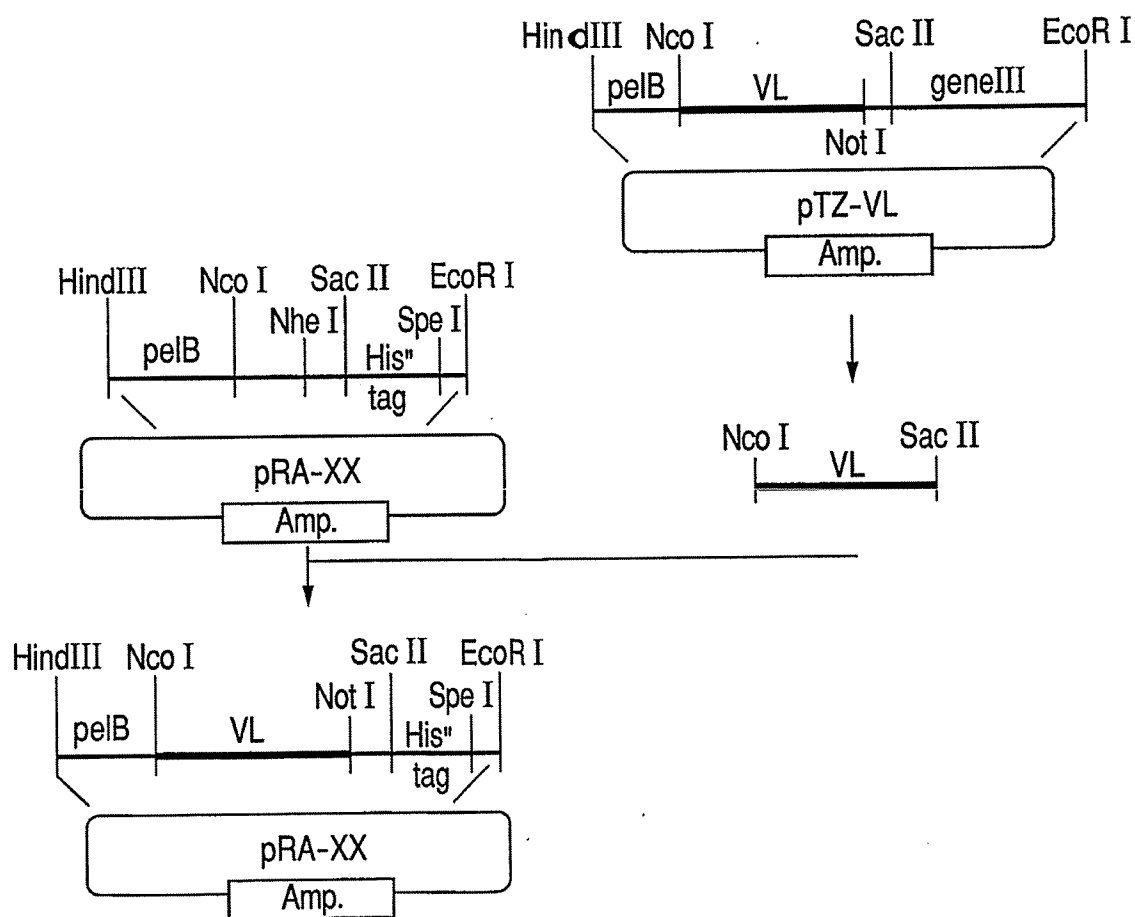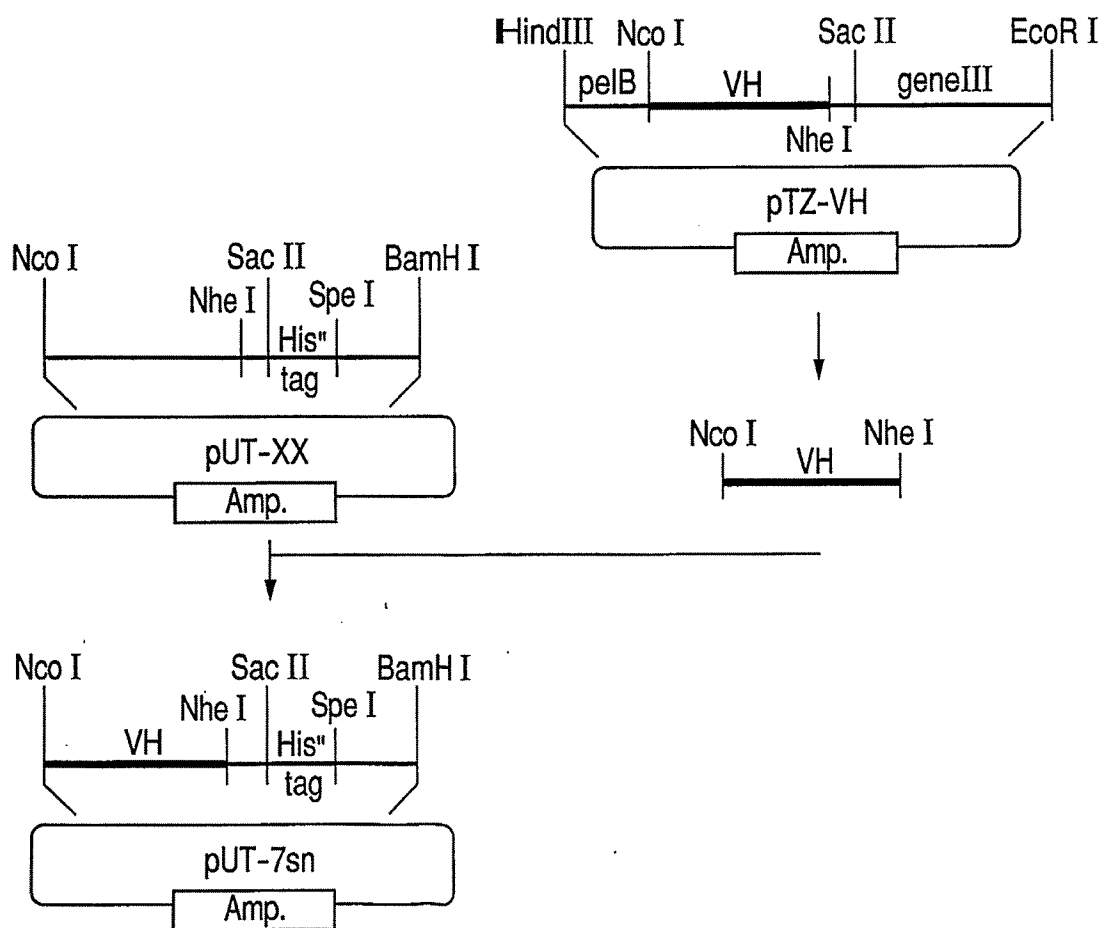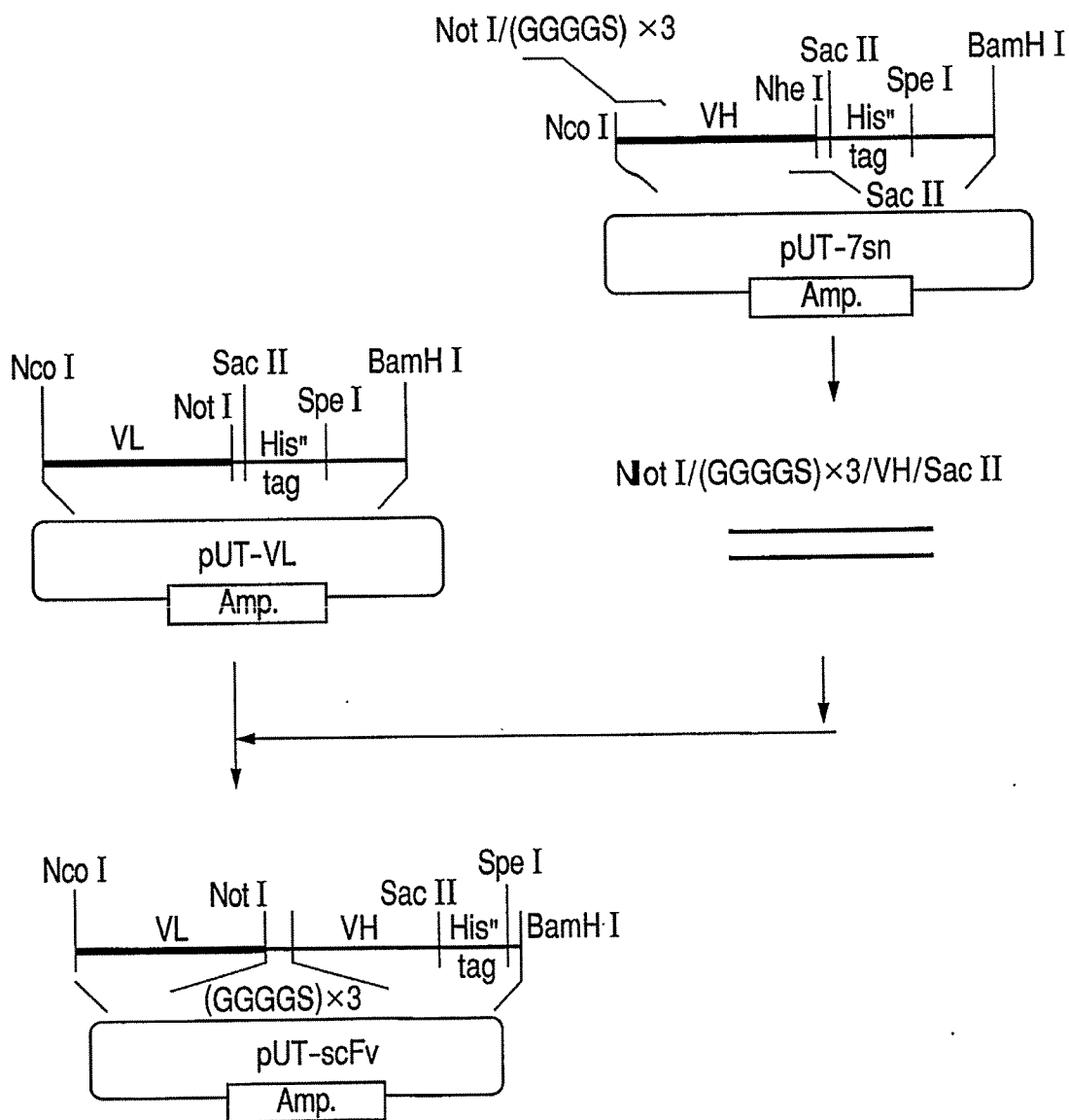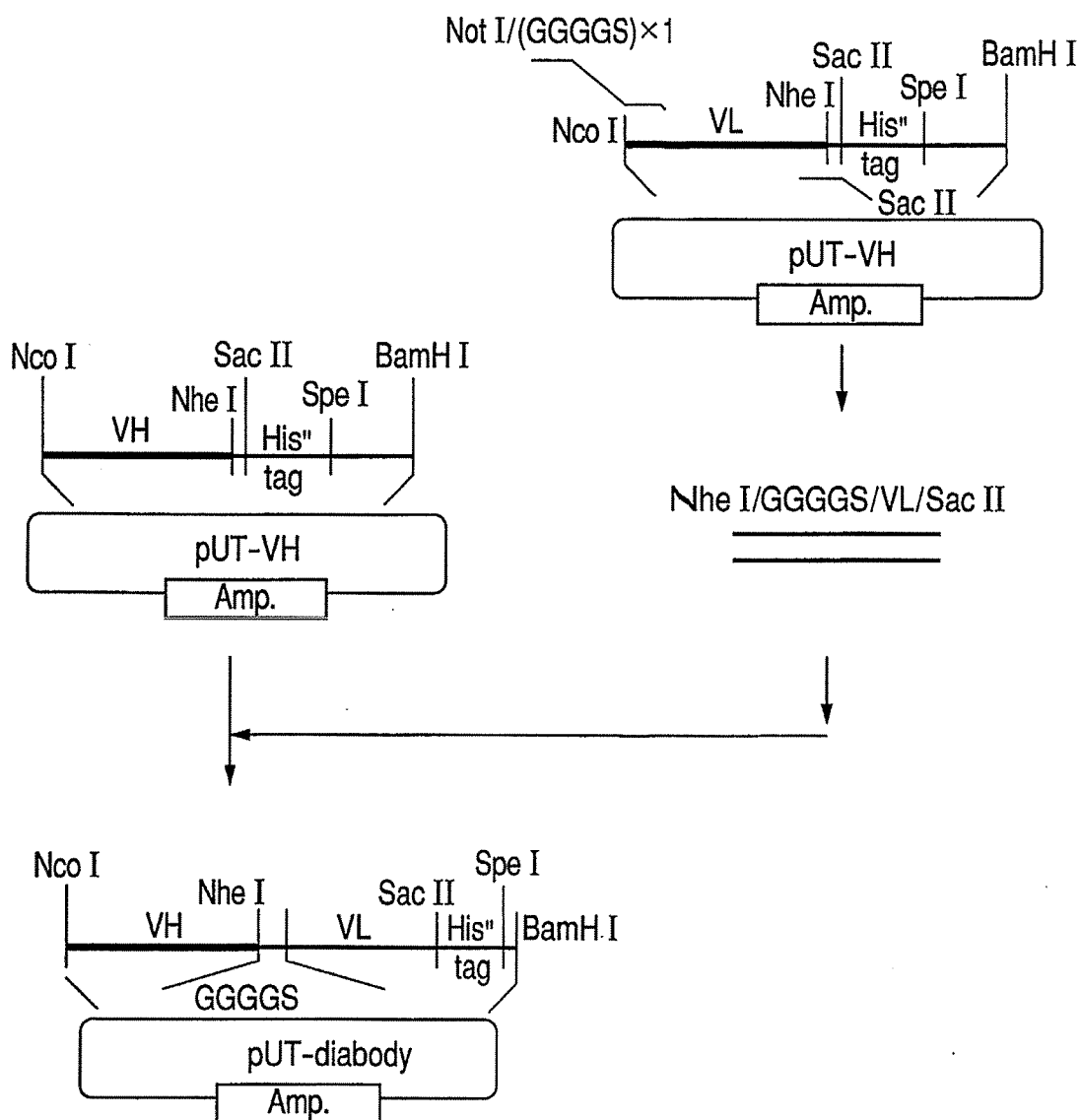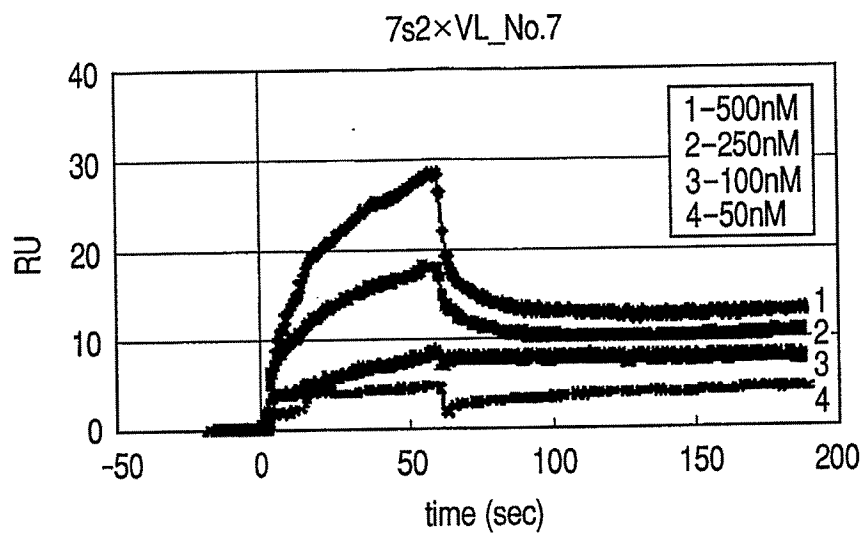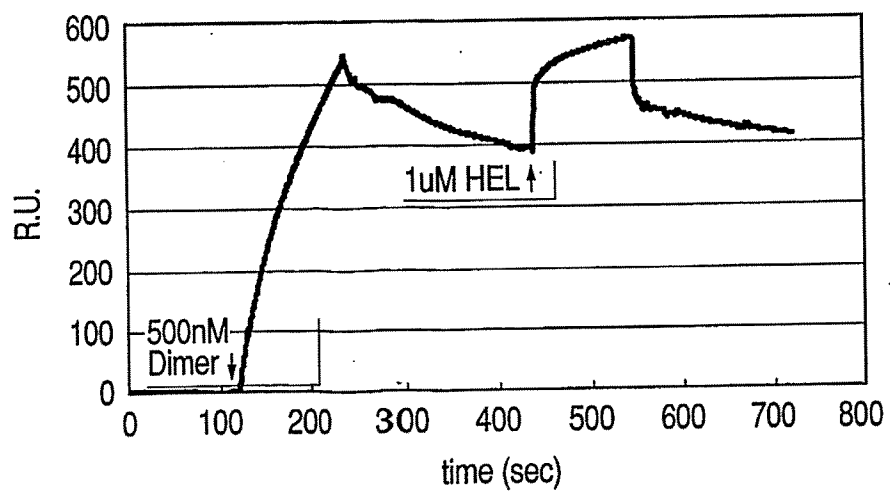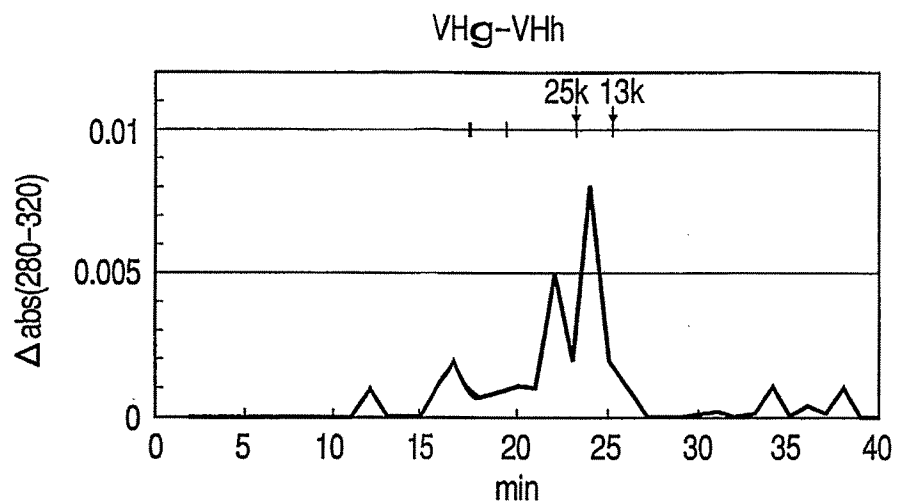
FIG. 13

FIG. 14

FIG. 15

FIG. 16

FIG. 17

7s2×VL_No.7

FIG. 18

FIG. 19

VHg-VHh

FIG. 20

FIG. 21

FIG. 22

FIG. 23

FER7×HGL7

FIG. 24

FIG. 25

FIG. 26

FIG. 27

500nM VGL+500nM BSA

FIG. 28

anti-HEL polyclonal → blocking → HEL
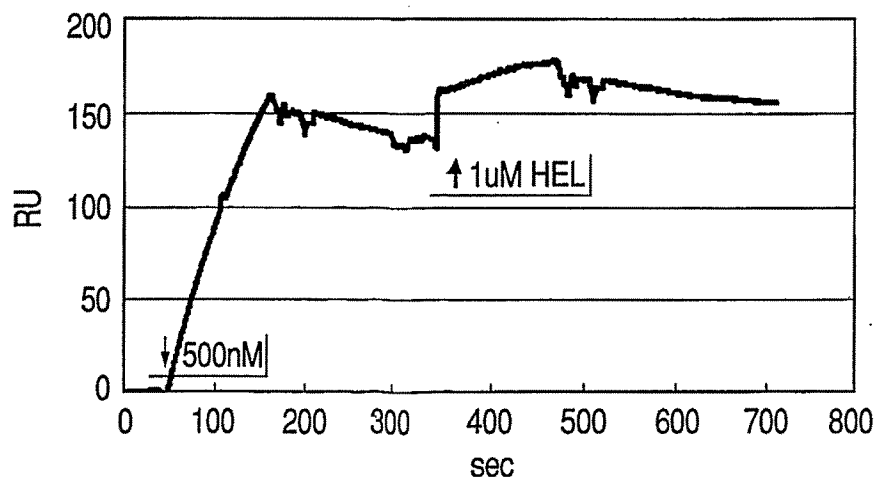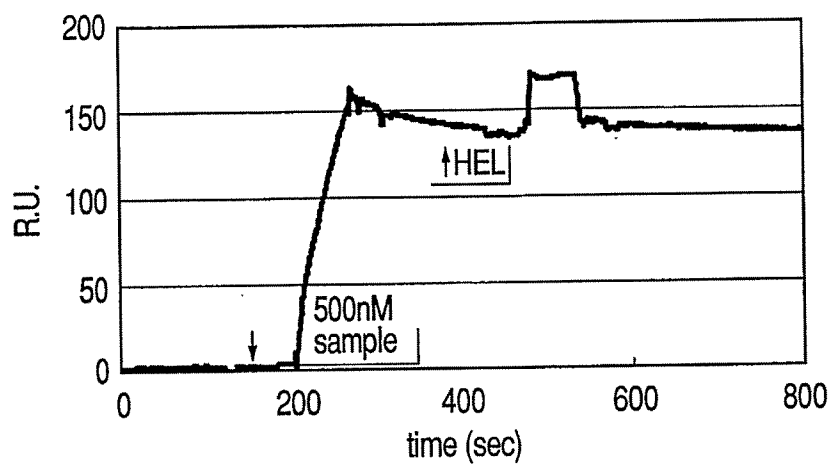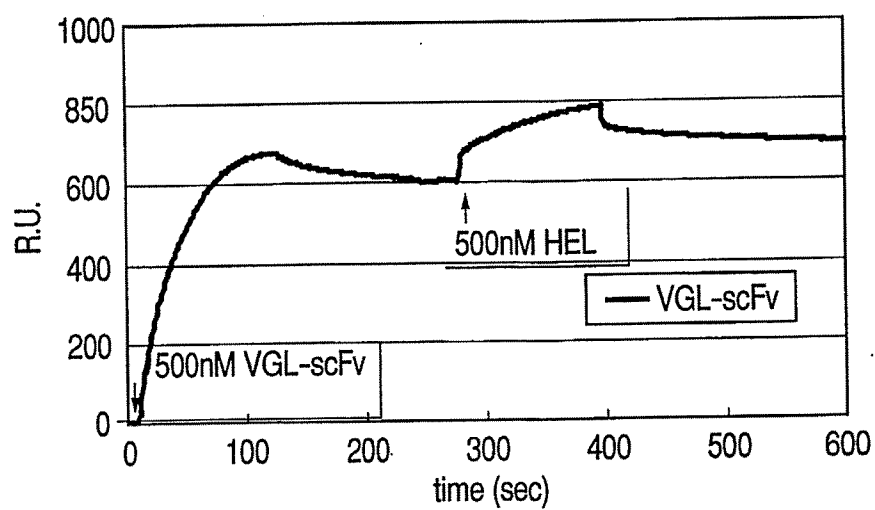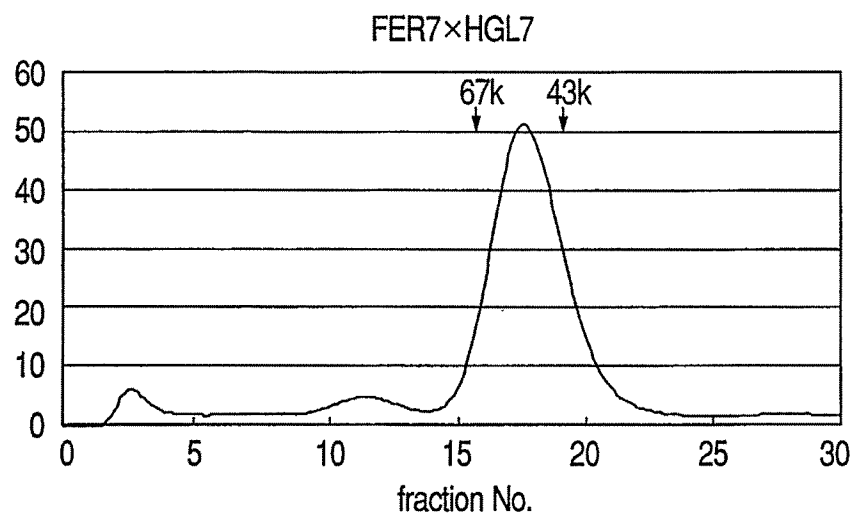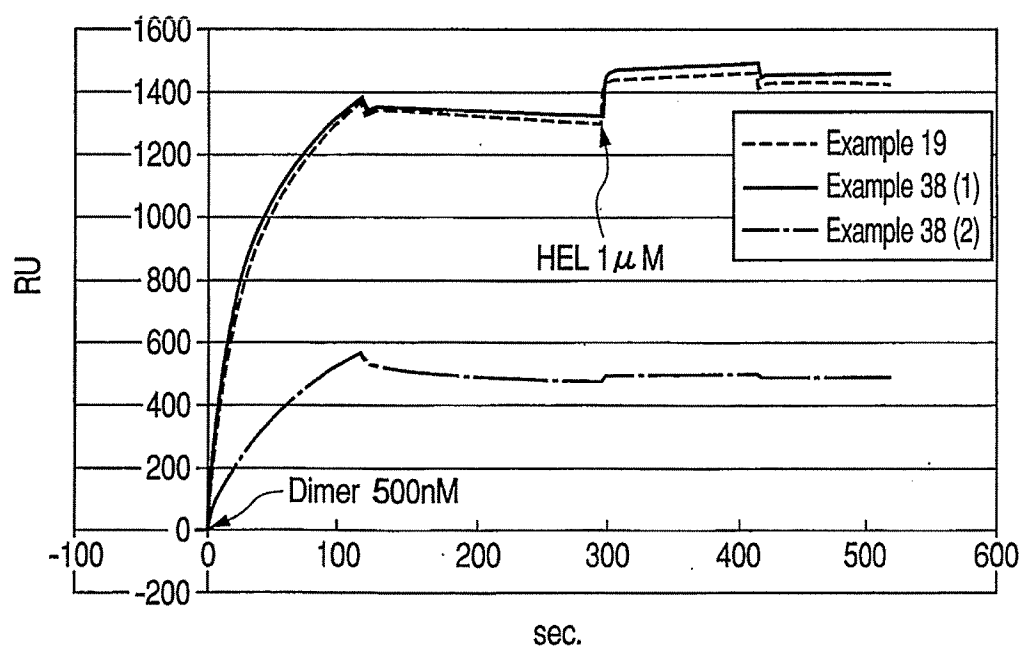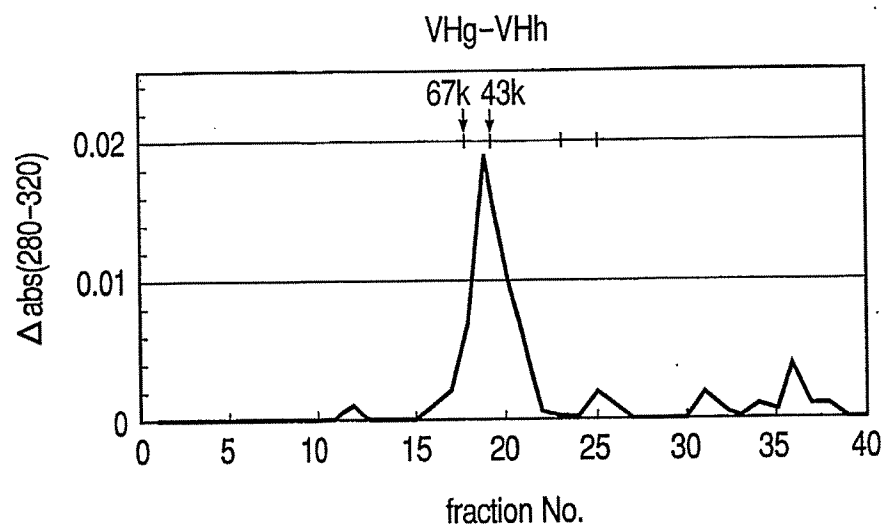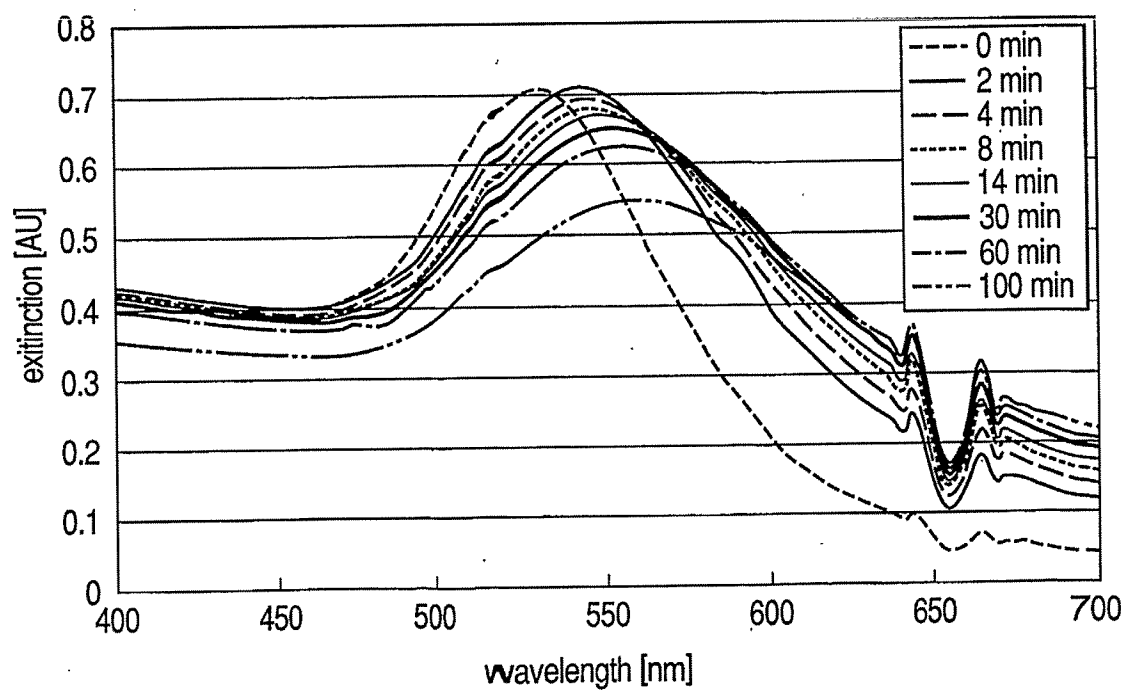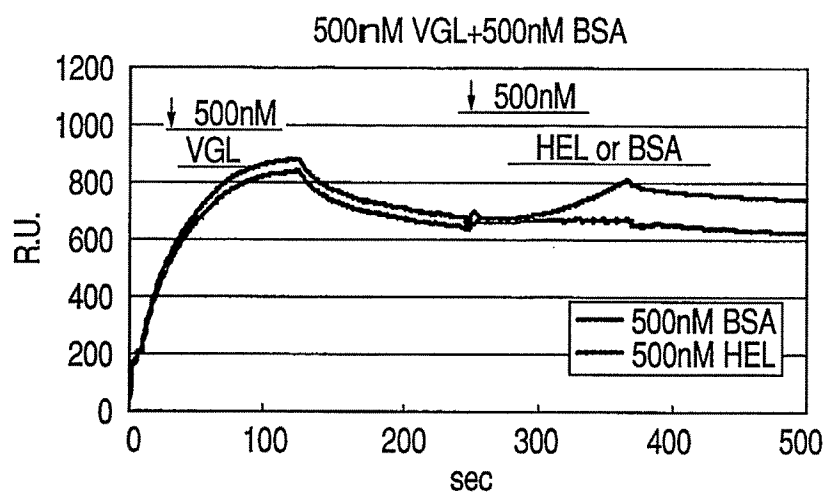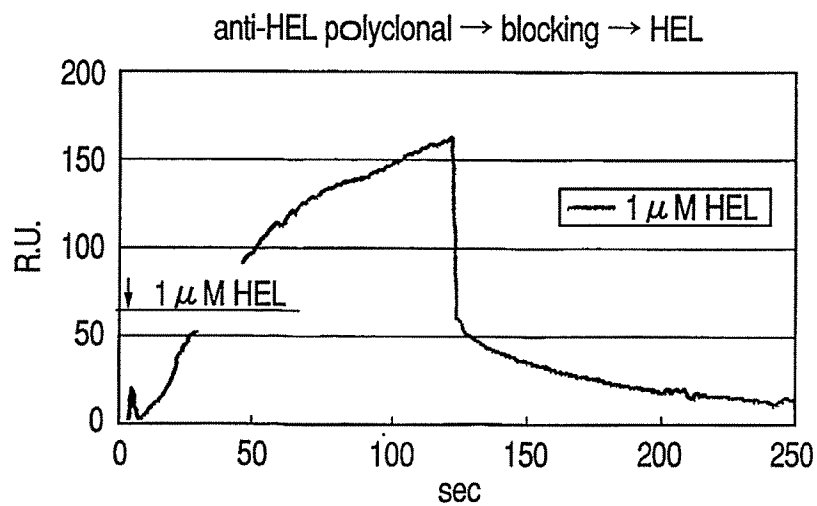
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<211> 12

<212> PRT

<213> human

<400> 37

Glu Leu Ile Thr Gly Arg Leu Pro Thr Asp Asn Asp

1   5   10

<210> 38

<211> 5

<212> PRT

<213> human

<400> 38

Gly Tyr Tyr Ile His

1   5

<210> 39

<211> 17

<212> PRT

<213> human

<400> 39

Trp Ile Asn Pro Asn Thr Gly Gly Thr Asn Tyr Ala Gln Lys Phe Gln

1   5   10   15

Gly

<210> 40

<211> 18

<212> PRT

<213> human

<400> 40

Arg Ser Gly Gly Ser Gly Arg Tyr Trp Gly Ile Lys Asn Asn Trp Phe

1   5   10   15

Asp Pro

<210> 41

<211> 17

<212> PRT

<213> human

<400> 41

Trp Ile Asn Pro Asn Ser Gly Val Thr His Tyr Ala Gln Lys Phe Gln

1   5   10   15

Gly

<210> 42

<211> 12

<212> PRT

<213> human

<400> 42

Glu Leu Ile Ala Gly Arg Leu Pro Thr Asp Asn Asp

1   5   10

<210> 43

<211> 5

<212> PRT

<213> human

<400> 43

Asp Tyr Tyr Phe His
1   5

<210> 44

<211> 17

<212> PRT

<213> human

<400> 44

Trp Ile Asn Phe Asp Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe Gln
1   5   10   15

Gly

<210> 45

<211> 13

<212> PRT

<213> human

<400> 45

Gly Ser Arg Tyr Asn Ser Gly Trp Tyr Tyr Phe Asp Tyr
1   5   10

<210> 46

<211> 5

<212> PRT

<213> human

<400> 46

Ile Ser Ser Pro Thr
1   5

<210> 47

<211> 16

<212> PRT

<213> human

<400> 47

Glu Ile Tyr His Ser Gly Thr Ser His His Asn Pro Ser Leu Lys Asn
1   5   10   15

<210> 48

<211> 11

<212> PRT

<213> human

<400> 48

Leu Asp Phe Asp Ser Pro Leu Gly Met Asp Ala
1   5   10

<210> 49
<211> 11
<212> PRT
<213> human

<400> 49

Arg Ala Ser Gln Ser Ile Ser Thr Tyr Leu Asn
1   5   10

<210> 50
<211> 7
<212> PRT
<213> human

<400> 50

Ala Ala Ser Ser Leu Gln Ser
1   5

<210> 51
<211> 9
<212> PRT
<213> human

<400> 51

Gln Gln Ser Tyr Ser Thr Pro Arg Thr
1   5

<210> 52
<211> 11
<212> PRT
<213> human

<400> 52

Arg Ala Ser Glu Asp Val Asn Ser Trp Leu Ala
1   5   10

<210> 53
<211> 7
<212> PRT
<213> human

<400> 53

Gly Ser Thr Asn Leu Gln Gly
1   5

<210> 54

<211> 10

<212> PRT

<213> human

<400> 54

Lys Tyr Phe Asp Ala Leu Pro Pro Val Thr
1   5   10

<210> 55

<211> 17

<212> PRT

<213> human

<400> 55

Thr Ser Ser Gln Ser Leu Leu Tyr Thr Ser Asn Asn Lys Asn Tyr Leu
1   5   10   15

Thr

<210> 56

<211> 7

<212> PRT

<213> human

<400> 56

Trp Ala Ser Thr Arg Glu Phe
1   5

<210> 57

<211> 9

<212> PRT

<213> human

<400> 57

Gln Gln Tyr Ser Asp Pro Pro Pro Thr
1   5

<210> 58

<211> 124

<212> PRT

<213> human

<233> 7s1

<400> 58

Glu Val Gln Val Val Glu Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1   5   10   15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Asn Ser His
            20   25   30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
            35   40   45

Gly Gly Phe Ile Pro Ile Phe Gly Thr Ser Asn Tyr Ala Glu Lys Phe
            50   55   60

Lys Gly Arg Val Thr Phe Thr Val Asp Thr Ser Thr Asn Thr Ala His
65   70   75   80

Met Glu Leu Thr Arg Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
                85   90   95

Ala Leu Pro Arg Arg Ser Ser Ser Ser Lys Thr Phe Ser Ala Leu Asp
            100   105   110

Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
            115   120

<210> 59
<211> 129
<212> PRT
<213> human
<233> 7s2
<400> 59

Gln Val Gln Leu Thr Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
1   5   10   15

Ser Leu Arg Leu Ser Cys Thr Ala Ser Gly Phe Thr Leu Gly Asp Tyr
            20   25   30

Ala Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
            35   40   45

Gly Phe Ile Arg Ser Arg Gly Phe Gly Gly Thr Pro Glu Tyr Ala Ala
            50   55   60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Gly Ile
65   70   75   80

Ala Tyr Leu Glu Met Asn Ser Leu Lys Thr Glu Asp Thr Ala Met Tyr
                85   90   95

Tyr Cys Thr Arg Asp Tyr Arg Pro Leu Gln Phe Trp Pro Gly Arg Gln

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                      100                105                110
Met Asp Ala Phe Asp Ile Trp Gly Gln Gly Thr Met Val Thr Val Sre
          115                120                125
Ser
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<210> 60
<211> 120
<212> PRT
<213> human
<233> 7s4
<400> 60
Gln Val Gln Leu Val Glu Ser Gly Ala Glu Val Lys Lys Ala Gly Glu
1               5                 10                15
Ser Leu Lys Ile Ser Cys Lys Gly Ser Gly Tyr Ser Phe Pro Ser Tyr
          20                25                30
Trp Ile Asn Trp Val Arg Gln Met Pro Gly Lys Gly Leu Glu Trp Met
          35                40                45
Gly Met Ile Tyr Pro Ala Asp Ser Asp Thr Arg Tyr Ser Pro Ser Phe
     50                55                60
Gln Gly His Val Thr Ile Ser Ala Asp Lys Ser Ile Asn Thr Ala Tyr
65               70                75                80
Leu Gln Trp Ala Gly Leu Lys Ala Ser Asp Thr Ala Ile Tyr Tyr Cys
               85                90                95
Ala Arg Leu Gly Ile Gly Gly Arg Tyr Met Ser Arg Trp Gly Gln Gly
          100                105                110
Thr Leu Val Thr Val Ser Ser Ala
          115                120
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<210> 61
<211> 119
<212> PRT
<213> human
<233> 7s7
<400> 61
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                 10                15
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Ser Val Lys Val Ser Cys Glu Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
              20   25   30
Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35   40   45
Gly Met Ile Asn Pro Arg Gly Gly Ser Thr Thr Tyr Ala Gln Lys Phe
    50   55   60
Gln Gly Arg Val Thr Met Thr Arg Asp Arg Ser Thr Ser Thr Val His
65   70   75   80
Met Glu Val Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
                85   90   95
Ala Arg Glu Ser Met Val Arg Asp Gly Met Asp Val Trp Gly Gln Gly
            100   105   110
Thr Thr Val Thr Val Ser Ser
        115

<210> 62
<211> 118
<212> PRT
<213> human
<233> 7s8
<400> 62
Glu Val Gln Leu Val Glu Ser Gly Ser Glu Val Lys Lys Pro Gly Ala
1   5   10   15
Ser Leu Lys Val Ser Cys Lys Ala Ser Gly Tyr Ser Phe Val Asp His
              20   25   30
Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35   40   45
Gly Trp Leu Asn Pro Asn Ser Gly Ala Thr Lys Tyr Ala Gln Lys Phe
    50   55   60
His Gly Arg Val Thr Leu Thr Arg Asp Thr Ser Ile Ser Thr Val Tyr
65   70   75   80
Met Glu Leu Ser Arg Leu Ile Ser Asp Asp Thr Ala Val Tyr Phe Cys
                85   90   95
Ala Arg Gly Ile Leu Leu Ala Arg Leu Asp Val Trp Gly Gln Gly Thr
            100   105   110
Leu Val Thr Val Ser Ser
        115

<210> 63

<211> 118

<212> PRT

<213> human

<233> 7p2

<400> 63

Gln Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val Gln Pro Gly Gly

1   5   10   15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr

          20   25   30

Tyr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Gln Trp Val

          35   40   45

Ala Ser Ile Lys Gln Asp Gly Ser Glu Thr Arg Tyr Gly Asp Ser Val

      50   55   60

Arg Gly Arg Phe Ile Ile Ser Arg Asp Asn Thr Lys Asn Ser Ala Tyr

65   70   75   80

Leu Gln Met Asn Thr Leu Ser Ala Glu Asp Arg Ala Val Tyr His Cys

              85   90   95

Val Arg Glu Leu Asp Gly Gly Phe Phe Asp Phe Trp Gly Gln Gly Thr

          100   105   110

Leu Val Thr Val Ser Ser

          115

<210> 64

<211> 121

<212> PRT

<213> human

<233> 7p3

<400> 64

Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu

1   5   10   15

Thr Leu Ser Leu Thr Cys Ser Val Ser Gly Ala Ser Met Ser Arg Phe

          20   25   30

Tyr Trp Asn Trp Ile Arg His Ser Ala Gly Lys Gly Leu Glu Trp Ile

          35   40   .   45

Gly Arg Ile Phe Thr Asn Gly Thr Thr Asn Tyr Asn Pro Ser Leu Gly

      50   55   60

Ser Arg Val Thr Met Ser Val Asp Thr Ala Lys Asn Gln Phe Ser Leu
65   70   75   80
Arg Val Thr Ser Val Thr Ala Ala Asp Ala Ala Val Tyr Tyr Cys Ala
                    85   90   95
Arg Gly Gly Asp Tyr Gly Pro Ala Leu Ala Trp Phe Asp Pro Trp Gly
                    100   105   110
Gln Gly Thr Leu Val Thr Val Ser Ser
                    115   120

<210> 65
<211> 124
<212> PRT
<213> human
<233> 7p4
<400> 65
Gln Val Gln Leu Gln Glu Trp Gly Gly Gly Val Val Gln Pro Gly Arg
1   5   10   15
Ser Leu Arg Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Gly His
                    20   25   30
Trp Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Val Trp Val
                    35   40   45
Ser Arg Ile Asp Glu His Gly Ser Ser Ala Tyr Tyr Ala Asp Ser Val
                    50   55   60
Asn Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65   70   75   80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                    85   90   95
Ala Arg Leu Gly Phe Ile Thr Pro Glu Val Val His Trp Ser Ser Asp
                    100   105   110
Ile Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
                    115   120
<210> 66
<211> 119
<212> PRT
<213> human
<233> 7p7

<400> 66

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1   5   10   15
Ser Val Lys Val Ser Cys Glu Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                20   25   30
Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
                35   40   45
Gly Met Ile Asn Pro Arg Gly Gly Ser Thr Thr Tyr Ala Gln Lys Phe
        50   55   60
Gln Gly Arg Val Thr Met Thr Arg Asp Arg Ser Thr Ser Thr Val His
65   70   75   80
Met Glu Val Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
                85   90   95
Ala Arg Glu Ser Met Val Arg Asp Gly Met Asp Val Trp Gly Gln Gly
                100   105   110
Thr Thr Val Thr Val Ser Ser
                115

<210> 67
<211> 128
<212> PRT
<213> human
<233> 7p8

<400> 67

Gln Val Gln Leu Gln Gln Trp Gly Ala Gly Leu Leu Lys Pro Ser Glu
1   5   10   15
Thr Leu Ser Leu Thr Cys Ala Val Tyr Gly Gly Ser Phe Ser Gly Tyr
                20   25   30
Tyr Trp Thr Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Ile
                35   40   45
Gly Asp Ser Ser His Ser Gly Arg Thr Asn Tyr Asn Pro Ser Leu Lys
        50   55   60
Ser Arg Val Thr Ile Ser Val Asp Ala Ser Lys Lys Gln Phe Ser Leu
65   70   75   80
Lys Leu Ile Ser Val Thr Ala Ala Glu Pro Phe Arg Ser Ser Ser Glu
                85   90   95
Met Ser Phe Cys Ser Leu Ala Glu Glu Thr Val Thr Ile Val Pro Trp

```
                 100             105             110
Pro Gln Thr Ser Lys Ala Pro Pro Asn Arg Pro Arg Cys Phe Val Ser
             115             120             125
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<210> 68
<211> 125
<212> ProArgT
<213> human
<233> 10s1
<400> 68

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Gly Ala Ala Gly Glu Ser Gly Ala Glu Val Arg Lys Pro Gly Ser Ser
1               5               10              15
Val Lys Val Ser Cys Lys Ala Ser Gly Gly Ser Ile Asn Asn Tyr Ala
             20              25              30
Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile Gly
         35              40              45
Gly Thr Leu Leu Met Leu Arg Ile Ile Asn Ser Ala Gln Lys Phe Gln
     50              55              60
Gly Arg Val Ser Ile Thr Ala Asp Thr Ser Thr Asn Thr Ala Tyr Met
65              70              75              80
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala
             85              90              95
Ser Ser Val Ala Ala Leu Pro Thr Ser Leu Gly Pro Ile Gly Tyr Leu
             100             105             110
His His Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
         115             120             125
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<210> 69
<211> 117
<212> PRT
<213> human
<233> 10s2
<400> 69

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Gly Ala Gly Gly Glu Ser Gly Ala Asp Val Lys Lys Pro Gly Ala Ser
1               5               10              15
Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr Tyr
             20              25              30
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Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly
            35   40   45
Trp Ile Asn Pro Asn Ile Gly Ala Thr Asn His Ala Gln Arg Phe Gln
      50   55   60
Gly Arg Leu Thr Val Ser Arg Asp Thr Ser Ile Thr Thr Val Tyr Met
65   70   75   80
Glu Leu Ser Arg Leu Gln Ser Asp Asp Thr Ala Val Tyr Phe Cys Ala
                  85   90   95
Arg Asp Leu Gly Ile Ser Ala Phe Glu Asn Trp Gly Gln Gly Thr Met
              100   105   110
Val Thr Val Ser Ser
            115

<210> 70
<211> 120
<212> PRT
<213> human
<233> 10s3
<400> 70
Gly Asp Gly Gly Glu Ser Gly Ala Glu Val Arg Lys Pro Gly Ala Ser
1   5   10   15
Val Lys Val Ser Cys Lys Ala Ser Arg Tyr Ser Phe Thr Asp Tyr Phe
              20   25   30
Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly
            35   40   45
Trp Ile Asn Pro Asn Ser Gly Val Thr His Tyr Ala Gln Lys Phe Gln
      50   55   60
Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ile Asn Thr Ala Tyr Met
65   70   75   80
Glu Val Ser Arg Leu Arg Tyr Asp Asp Thr Ala Val Tyr Tyr Cys Thr
                  85   90   95
Arg Glu Leu Ile Thr Gly Arg Leu Pro Thr Asp Asn Asp Trp Gly Gln
              100   105   110
Gly Thr Leu Val Thr Val Ser Ser
            115   120

<210> 71
<211> 126

<212> PRT

<213> human .

<233> 10s4

<400> 71

Gly Ala Ala Gly Glu Ser Gly Ala Glu Val Arg Lys Pro Gly Ala Ser
1   5   10   15

Val Lys Val Ser Cys Lys Thr Ser Gly Tyr Asn Phe Arg Gly Tyr Tyr
                20   25   30

Ile His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly
        35   40   45

Trp Ile Asn Pro Asn Thr Gly Gly Thr Asn Tyr Ala Gln Lys Phe Gln
    50   55   60

Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ile Asn Thr Ala Tyr Met
65   70   75   80

Glu Val Ser Lys Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys Ala
                85   90   95

Arg Arg Ser Gly Gly Ser Gly Arg Tyr Trp Gly Ile Lys Asn Asn Trp
                100   105   110

Phe Asp Pro Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115   120   125

<210> 72

<211> 120

<212> PRT

<213> human

<233> 10s5

<400> 72

Gly Asp Gly Gly Glu Ser Gly Ala Glu Val Arg Lys Pro Gly Ala Ser
1   5   10   15

Val Lys Val Ser Cys Lys Ala Ser Arg Tyr Ser Phe Thr Asp Tyr Phe
                20   25   30

Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly
        35   40   45

Trp Ile Asn Pro Asn Ser Gly Val Thr His Tyr Ala Gln Lys Phe Gln
    50   55   60

Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ile Asn Thr Ala Tyr Met

65   70   75   80
Glu Val Ser Arg Leu Arg Tyr Asp Asp Thr Ala Val Tyr Tyr Cys Thr
                    85   90   95
Arg Glu Leu Ile Ala Gly Arg Leu Pro Thr Asp Asn Asp Trp Gly Gln
                    100   105   110
Gly Thr Leu Val Thr Val Ser Ser
             115   120

<210> 73
<211> 122
<212> PRT
<213> human
<233> 10p1
<400> 73
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1   5   10   15
Ser Val Lys Val Ser Cys Lys Ala Ala Gly Tyr Thr Phe Thr Asp Tyr
                    20   25   30
Tyr Phe His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
             35   40   45
Gly Trp Ile Asn Pro Asp Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe
       50   55   60
Gln Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ile Ser Thr Ala Tyr
65   70   75   80
Met Asp Leu Ser Arg Leu Arg Ser Asp Asp Thr Ala Val Cys Tyr Cys
                    85   90   95
Ala Arg Gly Ser Arg Tyr Asn Ser Gly Trp Tyr Tyr Phe Asp Tyr Trp
                    100   105   110
Ser Arg Gly Thr Leu Val Thr Val Ser Ser
             115   120

<210> 74
<211> 120
<212> PRT
<213> human
<233> 10p2
<400> 74

Gln Val Gln Leu Gln Glu Trp Gly Pro Gly Leu Val Lys Pro Ser Gly
1   5   10   15

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Gly Thr Ile Ser Ser Pro
            20   25   30

Thr Trp Trp Asn Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp
        35   40   45

Ile Gly Glu Ile Tyr His Ser Gly Thr Ser His His Asn Pro Ser Leu
        50   55   60

Lys Asn Arg Val Thr Leu Ser Val Asp Lys Ser Lys Asn Gln Phe Ser
65   70   75   80

Leu Lys Leu Asn Ser Met Thr Ala Ala Asp Thr Ala Val Tyr Phe Cys
                85   90   95

Thr Arg Leu Asp Phe Asp Ser Pro Leu Gly Met Asp Ala Trp Gly Gln
            100   105   110

Gly Thr Thr Val Thr Val Ser Ser
            115   120

<210> 75

<211> 109

<212> PRT

<213> human

<233> No. 4

<400> 75

Asp Ile Val Met Thr Arg Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1   5   10   15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Thr Tyr
            20   25   30

Leu Asn Trp Tyr Gln Gln Lys Ala Gly Lys Ala Pro Lys Leu Leu Ile
            35   40   45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
        50   55   60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65   70   75   80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ser Tyr Ser Thr Pro Arg
                85   90   95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Ala
            100   105

<210> 76
<211> 110
<212> PRT
<213> human
<233> No.7
<400> 76
Glu Ile Val Leu Thr Gln Ser Pro Ser Ser Val Ser Ala Ser Val Gly
1   5   10   15
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Glu Asp Val Asn Ser Trp
            20   25   30
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
    . 35   40   45
Tyr Gly Ser Thr Asn Leu Gln Gly Gly Val Pro Ser Arg Phe Ser Gly
    50   55   60
Arg Gly Ser Gly Thr His Phe Thr Phe Thr Ile Asn Gly Leu Gln Pro
65   70   75   80
Glu Asp Ile Ala Thr Tyr Tyr Cys Lys Tyr Phe Asp Ala Leu Pro Pro
            85   90   95
Val Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys Arg Ala
            100   105   110

<210> 77
<211> 115
<212> PRT
<213> human
<233> No.10
<400> 77
Asp Ile Val Leu Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly
1   5   10   15
Glu Arg Ala Thr Ile Asn Cys Thr Ser Ser Gln Ser Leu Leu Tyr Thr
            20   25   30
Ser Asn Asn Lys Asn Tyr Leu Thr Trp Tyr Gln Gln Lys Pro Gly Gln
            35   40   45
Pro Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg Glu Phe Gly Val
    50   55   60
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr

```
        65              70              75              80
Ile Asn Ser Leu Gln Ala Glu Asp Val Ala Thr Tyr Tyr Cys Gln Gln
                     85              90              95
Tyr Ser Asp Pro Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
                    100             105             110
Lys Arg Ala
           115
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<212> DNA

<213> artificial sequence

<400> 78

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<212> DNA

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<400> 79

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<210> 80

<211> 52

<212> DNA

<213> artificial sequence

<400> 80

tcgcaactgc ggcccagccg gccatggccc agstrcagct gcagsagtcr gg 52

<210> 81

<211> 52

<212> DNA

<213> artificial sequence

<400> 81

tcgcaactgc ggcccagccg gccatggccs argtgcagkt ggtggagtct gg 52

<210> 82

<211> 52

<212> DNA

<213> artificial sequence

<400> 82

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<210> 83

<211> 52

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tcgcaactgc ggcccagccg gccatggccc aggtgcagct acagsagtgg gg 52

<210> 84

<211> 50

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<212> ProArgT

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<400> 85

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<210> 86

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attctcgact gctagctgag gagacggtga ccagggttcc 50

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<210> 88

<211> 22

<212> DNA

<213> artificial sequence

<400> 88

ccgctggatt gttattactc gc 22

<210> 89

<211> 52

<212> DNA

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<400> 89

tcgcaactgc ggcccagccg gccatggccg mcatycagwt gacccagtct cc 52

<210> 90

<211> 52

<212> DNA

<213> artificial sequence

<400> 90

tcgcaactgc ggcccagccg gccatggccg atrttgtgat gacycagwct cc 52

<210> 91

<211> 52

<212> DNA

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<400> 91

tcgcaactgc ggcccagccg gccatggccg aaattgtgwt gacgcagtct cc 52

<210> 92

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<210> 93

<211> 42

<212> DNA

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<400> 93

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<210> 94

<211> 52

<212> DNA

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<400> 94

ttctcgactt gcggccgcac gtttgatctc cagcttggtc cc 52

<210> 95

<211> 52

<212> DNA

<213> artificial sequence

<400> 95

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<210> 96

<211> 52

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<400> 96

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<210> 97

<211> 52

<212> DNA

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<400> 97

ttctcgactt gcggccgcac gtttaatctc cagtcgtgtc cc 52

<210>   98
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<213> human
<233> d7s1
<400> 98

gaggtgcagg tggtggagtc tgggggctgag gtgaagaagc ctggggcctc agtgaaggtt   60

tcgtgcaagg cttccggata caccttcaac agtcactata tccactgggt gcgacaggcc   120

cctggacaag ggcttgagtg gatggggggg ttcatcccta tctttggcac atcaaattac   180

gcagagaagt tcaagggcag agtcaccttt accgtggaca cgtccacgaa tacagcgcac   240

atggaactga ccagactgag atctgaggac acggccatat attactgtgc actaccccgg   300

aggagcagct cgtccaagac tttctcggcc cttgactact ggggccaggg caccctggtc   360

accgtctcct ca   372

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<211>   387
<212>   DNA
<213> human
<233> d7s2
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ccagggaagg ggctggagtg ggtaggtttc attcgaagca gaggtttcgg tgggactcca   180

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1   5   10   15

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Gly Tyr Val Ser Tyr Ser Gly Ser Thr Tyr Tyr Asn Pro Ser Leu Lys
        50   55   60

Ser Arg Ile Ser Ile Thr Arg Asp Thr Ser Lys Asn Gln Tyr Tyr Leu
65   70   75   80

Asp Leu Asn Ser Val Thr Thr Glu Asp Thr Ala Thr Tyr Tyr Cys Ala
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Asn Trp Asp Gly Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser
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Leu His Trp Tyr Gln Gln Lys Ser His Glu Ser Pro Arg Leu Leu Thr

        35   40   45

Lys Tyr Ala Ser Gln Ser Ile Ser Gly Thr Pro Ser Arg Phe Ser Gly

        50   55   60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser Ile Asn Ser Val Glu Thr

65   70   75   80

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<213> artifical sequence

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AGT TAC TGG ATC AAC TGG TTC CGC CAG ATG

CCC GGC AAA GAA AGG GAA TGG ATG GGG ATG

ATC TAT CCT GCT GAC TCT GAT ACC AGA TAT

AGC CCG TCC TTC CAA GGC CAC GTC ACC ATC

TCA GCC GAC AAG TCC ATC AAC ACC GCC TAC

CTG CAA TGG GCC GGC CTG AAG GCC TCG GAC

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S	C	K	G	S	G	Y	S	F	P
S	Y	W	I	N	W	F	R	Q	M
P	G	K	E	R	E	W	M	G	M
I	Y	P	A	D	S	D	T	R	Y
S	P	S	F	Q	G	H	V	T	I
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L	Q	W	A	G	L	K	A	S	D
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<212> PRT

<213> recombinant protein

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S V E T E D F G M Y F C . Q Q S N S W P Y T F G G

T K L I I T A G G G G S G G G G S G G G G S D I Q

L Q E S G P S L V K P S Q T L S L T C S V T G D S I

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G  S  T  Y  Y  N  P  S  L  K  S  R  I  S  I  T  R  D  T  S  K  N  Q  Y  Y  L

D  L  N  S  V  T  T  E  D  T  A  T  Y  Y  C  A  N  W  D  G  D  Y  W  G

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scFv-r

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